H_2O_2-mediated oxidation and nitration enhances DNA binding capacity / DNA repair via up-regulated epidermal wild-type p53 in vitiligo

Department of Biomedical Sciences,
Clinical and Experimental Dermatology,
University of Bradford

PhD Thesis

Mohamed Metwalli AbouElloof Salem

2009

Summary

The entire epidermis of patients with vitiligo exhibits accumulation of up to 10^{-3}M concentrations of hydrogen peroxide (H_2O_2) (Schallreuter, Moore et al. 1999). Over the last decade our group and others have focused on the effect of H_2O_2-mediated oxidative stress on the function of many proteins and peptides due to oxidation of target amino acid residues in their structure including L-methionine, L-tryptophan, L-cysteine and seleno cysteine (Rokos, Beazley et al. 2002; Gillbro, Marles et al. 2004; Hasse, Kothari et al. 2005; Schallreuter, Chavan et al. 2005; Spencer, Chavan et al. 2005; Chavan, Gillbro et al. 2006; Elwary, Chavan et al. 2006; Gibbons, Wood et al. 2006; Schallreuter, Bahadoran et al. 2008; Shalbaf, Gibbons et al. 2008; Wood, Decker et al. 2009). Moreover, it was shown that patients with vitiligo possess up regulated wild type functioning p53 protein in their skin (Schallreuter, Behrens-Williams et al. 2003). The reason behind this up regulation has remained unclear (Schallreuter, Behrens-Williams et al. 2003).

Therefore the aim of this thesis was to get a better understanding of these puzzling data. Along this project different techniques have been used including Western blot, dot blot, immuno precipitation, immuno fluorescence, EMSA and computer modelling.

In this thesis we confirmed the previous result on up regulation of p53 in vitiligo and we showed that $p90^{MDM2}$, the master regulator for p53 protein is not different in patients and healthy controls. Therefore we decided to test for expression of $p76^{MDM2}$ which mediates the inhibition of $p90^{MDM2}$-p53 binding. Our results show for the first time the presence and over expression of $p76^{MDM2}$ protein in vitiligo compared to

healthy individuals. This result could provide an explanation, why up regulated p53 is not degraded in this disease.

Since epidermal H_2O_2 accumulation has been extensively documented in vitiligo, we wanted to know whether other ROS could also contribute to the overall oxidative stress in this scenario. Therefore we turned our interest to nitric oxide (NO) and its possible effects on p53 protein. In order to elucidate this role in more detail, the expression levels of epidermal nitric oxide synthesase (iNOS) and the oxidation product of NO and O_2^- i.e peroxynitrite ($ONOO^-$) were investigated. Our data revealed over expression of iNOS and nitrated tyrosine residues, the foot print for $ONOO^-$. Moreover, we show for the first time the presence of abundant nitration of p53 protein in vitiligo. In addition using purified p53 from *E. coli* strain (BL21/DE3) and mutant p53 protein from HT-29 cells (colon cancer cells), we show that nitration takes place in a dose and time dependent manner. On this basis we investigated the effect of both H_2O_2 and $ONOO^-$ on p53-DNA binding capacity employing EMSA, since this is the most acceptable technique to follow the binding between proteins and DNA. Our results revealed that $ONOO^-$ abrogated p53-DNA binding capacity at concentrations >300 μM, meanwhile oxidation of p53 protein with H_2O_2 at the same concentrations does not affect binding capacity. Importantly, a much higher p53-DNA binding capacity was observed after exposure to both $ONOO^-$ and H_2O_2.

Taken together, p53 is regulated by both ROS (H_2O_2) and RNS ($ONOO^-$).

Next we identified the presence of phosphorylated and acetylated p53 in vitiligo. Phosphorylation of ser 9 and ser 15 residues of the protein are associated with over expressed ATM protein kinase, while acetylation of lys 373, 382 residues correlates with increased PCAF expression. We show that up regulated p53 is associated with over expressed p21 (cyclin dependent kinase inhibitor 1) and induced PCNA

expression. Hence, we can conclude that p53 in patients with vitiligo is up regulated, activated and functional.

Finally we show up regulated BCL-2 supporting the long voiced absence of increased apoptosis in vitiligo. Given that patients with vitiligo have no increased risk for solar induced skin cancer and increased photo damage (Calanchini-Postizzi and Frenk 1987; Westerhof and Schallreuter 1997; Schallreuter, Tobin et al. 2002), despite the presence of increased DNA damage as evidenced by increased 8-oxoG levels in the skin and in the plasma, our findings suggest that both p53 and PCNA provide a powerful machinery to mediate DNA repair via hOgg1, APE1 and DNA polymerase β (Shalbaf 2009). On this basis it is tempting to conclude that DNA-repair is the overriding mechanism to combat oxidative stress in this disease.

Acknowledgments

First and foremost, I would like to thank Professor K U Schallreuter for taking me as her student. She strongly supports her students and encourages them in doing research. She does not hesitate to give them the good advice and precise guidance during their study. Without her dedication and help, none of the work in this thesis would have been possible. Professor Schallreuter has a heart of gold and she is the most energetic person.

I would also like to thank Dr. J Thornton for being my second supervisor.

I am especially grateful to Professor Schallreuter's late husband, Professor J M Wood for providing help at any time. He was characterised with good listening, a nice smile and very useful advice. I would like also to thank Dr. H Rokos who was ready to help once I asked. At the same time I would like to thank Dr. N C J Gibbons for his co-operation by providing me with the molecular modelling which in turn gave a lot of explanation and good prediction during this study.

Moreover, many thanks to Dr. Jennifer Spencer, Dr. Giorgia Chiuchiarelli, Dr. Sybille Hasse, Dr. Bhaven Chavan, Mohammad Shalbaf and Christian Krüger for their kind dealing, advice and helping me during this period.

I would like to thank my lovely wife for her patience and steady encouragement. At the end I would like to thank my mother and my brothers, who supported me all the time.

The work for this PhD thesis was supported by the Egyptian government and by the Institute for Pigmentary Disorders in association with the EM Arndt University of Greifswald, Germany and University of Bradford, UK.

TABLE OF CONTENTS PAGE

List of Figures and Tables

List of abbreviations

AC	acetylation
AMPK	5'AMP-activated protein kinase
Ap	ampicillin
APAF1	apoptotic protease-activating factor 1
Arg	arginine
Asn	asparagine
ATM	ataxia telangiectasia mutated kinase
ATP	adenosine 5'-triphosphate
ATR	ataxia telangiectasia related kinase
BAX	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
$6BH_4$	(6R)-L-erythro 5, 6, 7, 8 tetrahydrobiopterin
BID	BH3 interacting domain death agonist
BSA	bovine serum albumin
CAK	Cdk-activated kinase
CDKs	cycline dependent kinases
cDNA	complementary DNA
CHK2	check point kinase 2
CHK1	check point kinase 1
CKI	casine kinase I
CKII	casine kinase II
CSB	Cockayne's group B
DAPI	4, 6-diamino-2-phenylindole
DBD	DNA binding domain
DD	dead domain
DDT	dithiothreitol
DISC	death inducing signalling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNA-PK	DNA-dependent protein kinase
ds	double DNA strand break
e NOS	endothelial nitric oxide synthase
E.*Coli*	Escherichia coli
ECL	enhanced chemiluminescence
EDRF	endothelium-derived relaxing factor
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
F^{19}	phenylalanine 19
FADD	FAS receptors dead domain
FAS	death receptor protein
FASL	FAS ligand
FITC	fluorescein isothiocyanate
FT	Fourier transform
Gadd45	growth arrest DNA damage inducible 45
GC	guanylyl cyclase
GMP	guanosine monophosphate
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-α	hypoxia inducible factor-α
His	histidine
HRP	horse radish peroxidase
HSB	high salt buffer
IAP	inhibitory apoptotic protein
IgG	immunoglobulin G
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	ionizing radiation
JNK	jun N-terminal kinase
kDa	kilo Dalton
L^{22}	leucine 22

LB	Luria-Bertani media
LFL	Li-Fraumeni like syndrome
LFS	Li-Fraumeni syndrome
Lps	lipopolysaccharide
Lys	lysine
MAPK	mitogen-activated protein kinase
MDM2	mouse double minute
Met	methionine
mRNA	messenger ribonucleic acid
αMSH	alpha melanocyte stimulating hormone
MW	molecular weight
nNOS	neural nitric oxide synthase
NAC	N-acetyl cysteine
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NRD	negative regulatory domain
NDS	normal donkey serum
NLS	nuclear localisation signal
NO	nitric oxide
NOS	nitric oxide synthase
$O_2{\cdot}^-$	superoxide anion
OCT	optimal cutting temperature compound
OD	optical density
$OH^{\cdot}$	hydroxyl radical
oligo	oligomerization domain
P14ARF	alternate reading frame
P^{21}	cyclin-dependent kinase inhibitor
PCAF	histone acetyltransferase (HAT)
PBS	phosphate buffered saline
PCD	pterin 4a-carbinolamine dehydratase
PC-KUS	pseudocatalase K U Schallreuter
PCNA	proliferating cell nuclear antigen

PDGF	platelet derived growth factor
PKC	protein kinase c
PMSF	phenyl-methyl-sulphonyl-fluoride
PN&ONOO$^-$	peroxynitrite
POMC	pro-opiomelanocortin peptide
PUMA	p53 upregulated modulator of apoptosis
RE	response elements
RB	retinoblastoma protein
REG	regulatory domain
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPA	replicating protein antigen
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodeyl sulphate polyacrylamide gel electrophoresis
Ser	serine
ss	single DNA strand break
SV40	simian virus 40
TAD	transactivation domain
TAF	TBP-associated factor
TBP	TATA box binding protein
TET	tetramerization domain
TFIID	TATA box binding protein-associated factors ($TF_{II}D$)
TGF-β	transforming growth factor
TNFα	tumour necrosis factor alpha
TRITC	tetramethyl rhodamine isothiocyanate
TRP-1&2	tyrosine related protein 1&2
Tween 20	polyoxyethylenesorbitanmonolaureate
TYR	tyrosinase
Val	valine
UVA	ultraviolet A
UVB	ultraviolet B
UVR	ultraviolet radiation

W^{23}	tryptophan 23
WT	wild type
XP B&D	xeroderma pigmentosum group B&D

1.0 INTRODUCTION

1.1 p53 tumour suppressor gene

The p53 tumour suppressor has been one of the most studied genes in the last 28 years, because of its major role in cancer prevention. It has been reported that the malfunction of p53 pathway is a common hallmark for human tumours (Vogelstein, Lane et al. 2000; Vousden and Lane 2007). Therefore it was reviewed that the somatic mutation of *p53* gene is one of the most universal mechanism by which the function of p53 protein is affected in cancer progression (Petitjean, Mathe et al. 2007). Moreover, the p53 mutations are divided into tumour associated mutations which are present in somatic cells as a result of DNA damage and this mutation appears in the DNA-binding domain of p53. Germline mutations are detected in people with Li-Fraumeni syndrome giving a high risk for developing lots of tumours such as sarcoma, breast and brain cancer. However, the germline mutation causes silent mutations (Whibley, Pharoah et al. 2009). Importantly p53 has been recognised to be mutated and/or deleted in approximately 50% of human cancers (Hollstein, Sidransky et al. 1991). P53 has been also named the guardian of the genome (Lane 1992).

The *p53* gene is located on the short arm of chromosome 17 (Oren 1985) at 17p13.1, a region which is frequently deleted in human cancers. The protein was first identified as a 53 kDa protein that co-immunoprecipitated with the SV40 large T antigen in SV40 transformed cell lines (Lane and Crawford 1979; Agoff, Hou et al. 1993). Moreover, p53 was also easily detected in cancer cells by using antibodies specific for the protein. Subsequently, the *p53* gene was demonstrated to be an oncogene, because of its ability to increase plating efficiency of primary cultures

after transfection (Jenkins, Rudge et al. 1984) and after co-transfection with an activated *H-ras* oncogene in transformed primary rat embryo fibroblasts (Finlay, Hinds et al. 1988). However, it was later discovered that the *p53* gene is actually a tumour suppressor gene, since introduction of wild type *p53* gene stopped the formation of tumours (Finlay, Hinds et al. 1988) and blocked the ability of an oncogene, such as *H-ras,* to promote cancer. Further evidence for the *p53* gene to be a human tumour suppressor gene was provided (Chen, Chen et al. 1990). It was reported that the transfection of a wild type *p53* gene into human oesteosarcoma cells lacking endogenous *p53 gene* stopped neoplastic growth by reducing both cell growth and cell division. These data also suggested that the wild type *p53* gene was dominant over the mutant *p53* gene in the short-term.

In normal cells p53 is present at a very low concentration at approximately 1000 molecules/cell, and is therefore undetectable by antibodies, using standard immunocytochemistry or immunohistochemistry. In some cancer cells p53 protein is, however, readily detected because its half - life is extended from ~20 mins (WT p53) to ~18hrs (mutant p53 protein). P53 protein expression is regulated at the post-translational level which is consistent with mutant p53 being a tumour antigen (Momand, Zambetti et al. 1992; Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). P53 is also the founding member of a small family of proteins which includes two other members, p63 and p73. These two genes *p63* and *p73* (Arrowsmith 1999; Levrero, De Laurenzi et al. 1999; Levrero, De Laurenzi et al. 2000) are able to produce proteins (p63 and p73) which appear similar in structure and function but not identical to p53 protein (Kaghad, Bonnet et al. 1997; Yang, Kaghad et al. 1998). Both p63 and p73 proteins have the ability to bind to p53 response elements, activate p53 mediated gene expression and they are capable to promote apoptosis. But in

contrast to p53, both p63 and p73 are rarely mutated in cancer. They are playing a vital role in normal development (Mills, Zheng et al. 1999; Yang, Walker et al. 2000). The overall sequence homology and domain structures, for these three proteins, is shown in **Figure 1**.

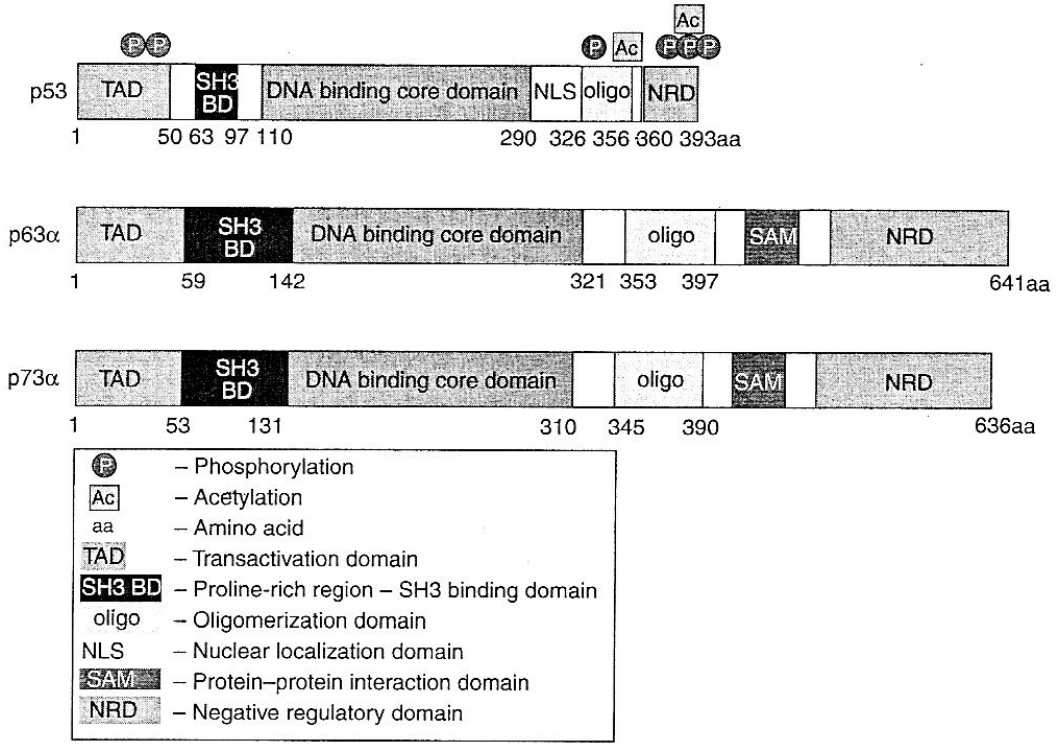


Figure 1
Structure of the p53 family
All three proteins (p53, p63 and p73) share common domains but they have different lengths for the NRD region (negative regulatory domain). Adapted from Pelengaris and Khan 2006.

1.2 Structure of p53 protein

P53 acts as a DNA-damage-inducible transcription factor, inducing cell cycle arrest, DNA repair and programmed cell death (Hollstein, Sidransky et al. 1991; He, Brinton et al. 1993; Lane 1993; Oren 1994; Prokocimer and Rotter 1994; Fukasawa, Choi et al. 1996; Ko and Prives 1996).

The structure of p53 contains 393 amino acids with an apparent molecular weight of 53kDa (Lane and Crawford 1979). P53 is activated in response to many cellular stresses, including DNA damage, ionizing radiation, viral infection, and hypoxia. P53 becomes stable and is present in high levels when activated by cellular stress (Kastan, Zhan et al. 1992; Lu and Lane 1993). Stabilization of p53 is associated with DNA binding activity and transcriptional activation of a number of other genes which mediate cell cycle arrest or apoptosis (Kastan, Zhan et al. 1992). In many human cancers, *p53* is mutated or loses its function as a tumour suppressor gene (Hollstein, Sidransky et al. 1991). The loss of *p53* function leads to a failure in cell cycle arrest, and repairing DNA damage, yielding cells with aberrant DNA which in turn gives rise to tumour growth, (reviewed in Lane 1992). P53 acts as a transcription factor, and contains the typical domain found in these proteins. There are five major domains which are very important for p53 function. These include: **1.** the N-terminal transcriptional activation domain, aa 1-42 (Unger, Juven-Gershon et al. 1999), **2.** a proline rich region aa 63-97 containing five repeats of the SH_3 binding motif PxxP (Walker and Levine 1996), **3.** the central sequence-specific DNA binding domain aa 102-292 (Bargonetti, Manfredi et al. 1993; Halazonetis, Davis et al. 1993; Pavletich, Chambers et al. 1993; Wang, Reed et al. 1993), **4.** The C-terminal which includes an oligomerization or tetramerization domain aa 323-356 and **5.** a regulatory domain aa

360-393 (Wang, Reed et al. 1993; Bakalkin, Yakovleva et al. 1994; Bayle, Elenbaas et al. 1995; Lee, Elenbaas et al. 1995; Reed, Woelker et al. 1995). Key parts of two of these domains are represented in five highly conserved regions in vertebrate species, these regions are, I aa 13-23, Π aa 117-142, III aa 171-181, IV aa 234-250aa, V aa 270-286 (Soussi, Caron de Fromentel et al. 1990; Soussi and May 1996), **(Figure 2).**

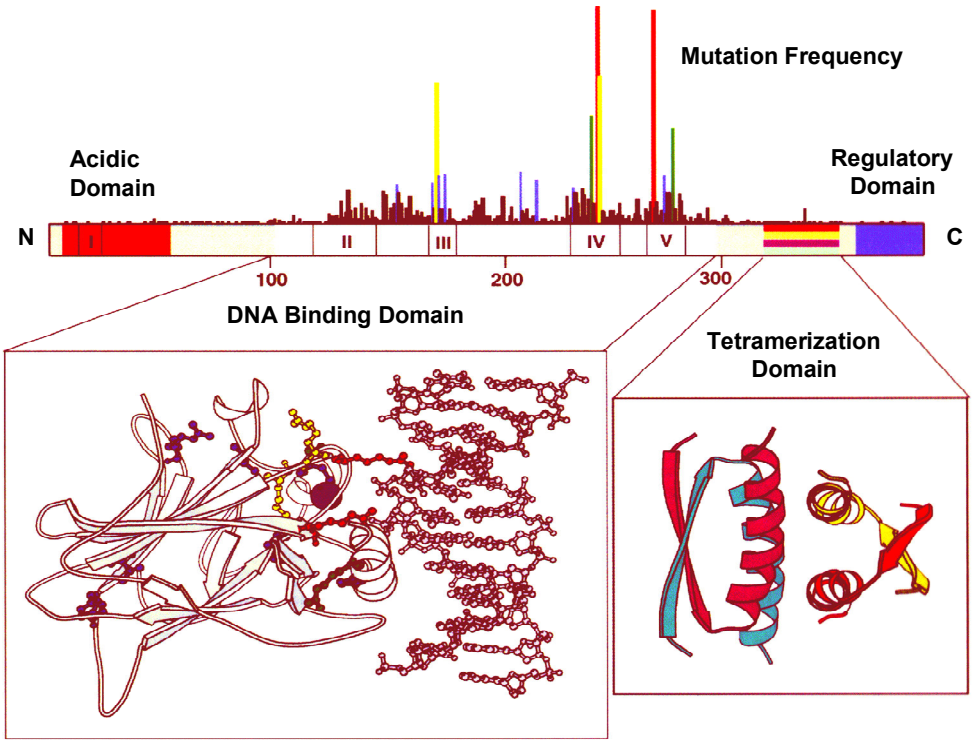


Figure 2
p53 containing the three dimensional structure of the DNA-binding domain complexed to DNA and the tetramerization domain
ß-strands are shown as arrows and α-helices as coils. The five conserved regions are shown in Roman numbers. The frequency of p53 mutations (hot spots) are indicated by a histogram along the top of p53 structure. Adapted from Arrowsmith and Morin 1996

1.2.1 The N-terminal domain of p53

This region is characterised by containing acidic amino acid residues. The N-terminal domain is a transcriptional activation domain, consisting of aa 1-42, which can enhance transcription by containing the binding sites for TATA box binding protein (TBP) and TATA box binding protein-associated factors ($TF_{II}D$) (Lu and Levine 1995; Thut, Chen et al. 1995). Both TBP and $TF_{II}D$ are positive factors for gene expression. It was reported that the N-terminal domain also binds to the transcription factor $TF_{II}D$ (Dutta, Ruppert et al. 1993; He, Brinton et al. 1993; Li and Botchan 1993). This region is also bound by proteins that negatively regulate the transcriptional activity of p53 (Momand, Zambetti et al. 1992; Yew and Berk 1992; Oliner, Pietenpol et al. 1993; Picksley, Vojtesek et al. 1994) namely the cellular protein mdm2, and the Ad E1B viral protein. All of these proteins bind p53 at amino acids sequence 13-23, playing an important role in transcription. This importance is underlined by the fact that mdm2 is the master regulator of p53 function, and negatively regulates its expression (Lin, Chen et al. 1994; Picksley, Vojtesek et al. 1994). Key residues in this region have been identified to be amino acids F^{19}, L^{22} and W^{23} (Lin, Chen et al. 1994; Picksley, Vojtesek et al. 1994). Beyond the transactivation domain (63-97) there is a hydrophobic proline rich region, aa 63-97, consisting of five copes of the sequence (PxxP). This region interacts with proteins having an -SH_3- domain (Src tyrosine kinase) and it has a role in the non-transcriptional activation of the intrinsic apoptotic pathway (Sakamuro, Sabbatini et al. 1997; Venot, Maratrat et al. 1998).

1.2.2 DNA binding domain of p53

The central domain of p53 contains the sequence specific for DNA binding. It is located between amino acids 102-292 (Hupp, Meek et al. 1992). This region is defined by its resistance to the protease thermolysin (Pavletich, Chambers et al. 1993). The DNA binding region of p53 has the ability to interact with specific DNA sequences in those genes which are regulated by p53. They are so called p53 response elements (RE). Cho et al revealed by X-ray crystallography that the DNA binding domain contains two large anti-parallel β sheets, each one having four and five β strands, forming a scaffold for 3 loops, L1, L2 and L3, which interact directly with the DNA duplex (Cho, Gorina et al. 1994). The interaction with DNA is as follows: loop L1 interacts with the major groove and the second loop L2 with the minor groove. The third loop L3 connects to L1 and co-ordinates a central Zn^{+2} atom. Because of the importance of the DNA binding domain, most point mutations occur within this region and are located within codons 126-307 of the *p53* gene. Hot spots at aa 175, 245, 248, 294, 273 and 282 play a role in the structural integrity of this domain for DNA contact sites **(Figure 3)**. It was demonstrated that p53 acts as a tetramer and mediates transcription by binding to a specific sequence (response element) located in the regulated region of the mediated genes (Raycroft, Wu et al. 1990; el-Deiry, Tokino et al. 1993; Cho, Gorina et al. 1994). These response elements consist of two halves, each half includes two copies of the sequence Pu Pu Pu C (A/T) (T/A) G py py py and this sequence is arranged by head to head and separated by 0-13 nucleotides (el-Deiry, Kern et al. 1992; Funk, Pak et al. 1992; Levine 1997). It was discovered that p53 response elements are present in many genes such as *p21*, *gadd45*, *mdm2* and *bax* (Ko and Prives 1996).

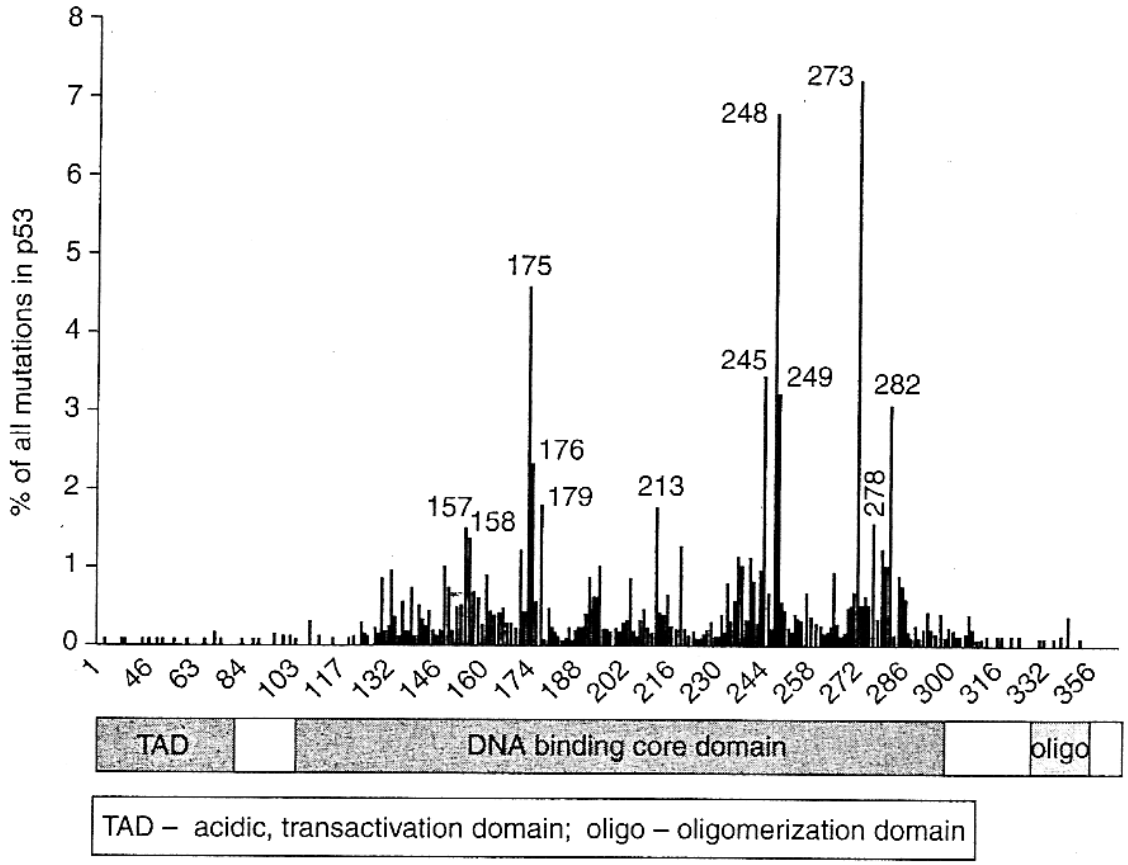


Figure 3
Locations for mutations of p53 in human cancer
Mutations are found along the entire sequence but there are high frequencies in the DNA-binding domain. Adapted from Pelengaris and Khan 2006.

1.2.3 Structure of the C-terminal domain

The C-terminal domain (aa 292-393) is protease-sensitive. The C-terminal domain contains a flexible linker (aa 300-318), a region responsible for oligomerization or tetramerization (amino acids 323-356) and a regulatory domain (amino acids 363-393) (Hupp, Meek et al. 1992). The amino acid residues 323-356 are responsible for the tetramerization or oligomerization of p53 (Kraiss, Quaiser et al. 1988). The C-terminal region regulates the sub-cellular localization of p53 according to three nuclear localization signals (NLS). Therefore, a mutation in NLS1 (amino acids 316-325) causes p53 to locate in the cytoplasm, while in NLS2 (amino acids 369-375) and in NLS3 (amino acids 379-384) leads to location in both cytoplasm and nucleus (Shaulsky, Goldfinger et al. 1991). The binding of monoclonal antibody PAb421 (which recognises a C-terminal epitope) of p53 can activate the DNA binding activity of p53 (Hupp, Meek et al. 1992). It was revealed that PAb421 antibody has the ability to restore the DNA binding activity of mutant p53 *in vitro* and *in vivo* (Dornan and Hupp 2001). Also the phosphorylation at residue Ser378 by casein kinase II inactivates the regulatory domain of p53 and the deletion of the C-terminal activates p53 DNA binding (Hupp, Meek et al. 1992).

1.3 Activation of p53

1.3.1 Phosphorylation of p53

Phosphorylation of proteins has been demonstrated to affect nuclear import or export of many proteins including SV40 large T antigen (Rihs and Peters 1989; Jans, Ackermann et al. 1991), cycline B1 (Yang, Bardes et al. 1998) and MAPKAP kinase 2 (Engel, Kotlyarov et al. 1998). Phosphorylation is important for p53 stabilization to perform its usual function as a transcription factor, (Giaccia and Kastan 1998; Appella and Anderson 2001). In this context it was shown that this process is induced by many stress factors including DNA damage affecting mainly 30 amino acids residues at both the amino terminus and the carboxyl terminus (Ito, Lai et al. 2001). It is known that in normal cells p53 is regulated by mdm2 in response to the genotoxic stress, such as DNA damage. P53 is hyperphosphorylated at multiple sites within or near the mdm2 binding domain (Giaccia and Kastan 1998; Chehab, Malikzay et al. 1999). The phosphorylation of p53 is related to cellular stresses, activating the expression of ATM and ATR (family of protein kinases) which mediate the phosphorylation of Ser 15 and Ser 37 on p53 (Canman, Lim et al. 1998; Tibbetts, Brumbaugh et al. 1999). It was shown by several groups (Shieh, Ikeda et al. 1997; Unger, Juven-Gershon et al. 1999; Hirao, Kong et al. 2000; Shieh, Ahn et al. 2000) that DNA damage causes the activation of CHK2 kinase promoting phosphorylation of p53 at ser 20 and ser 15 which in turn leads to its stabilization preventing mdm2 from binding to p53. Moreover, phosphorylation at ser15 of p53 mediates its binding to p300/CBP (histone acetyltransferase), where dephosphorylation leads to a defect in the activity of p53 (Lambert, Kashanchi et al. 1998; Dumaz and Meek 1999). Here it is noteworthy that DNA damage induces phosphorylation of p53 at a number of serine residues (i.e 6, 9, 15, 20, 33, 37 and

392). Recent studies have shown that the phosphorylation of ser 46 of p53 promotes the transactivation of proapoptotic genes (Shmueli and Oren 2007). For instance the mutation in ser 46 decrease the ability of p53 to activate proapoptotic genes such as Noxa and PUMA (Oda, Arakawa et al. 2000; Feng, Hollstein et al. 2006). DNA damage also mediates dephosphorylation of ser 376 leading to activation of the 14-3-3 protein, a large family of protein (28-33 kDa) causing cell cycle arrest (Knippschild, Milne et al. 1997; Shieh, Ikeda et al. 1997; Siliciano, Canman et al. 1997; Waterman, Stavridi et al. 1998). Phosphorylation of p53 at ser 392 mediates p53 tetramer formation. P53 is phosphorylated at ser37 by DNA-PK (DNA-protein kinase) (Shieh, Ikeda et al. 1997). All phosphorylation sites are shown in **Figure 4**.

1.3.2 Acetylation of p53

Acetylation is one of the posttranslational modifications for many proteins. Acetylation occurs for 85% of eukaryotic proteins (Polevoda and Sherman 2000). p300/CBP or PCAF a histoneacetyltransferase (HAT) acetylates lysine residues (i.e lys-370, lys-371, lys-372,373, lys-381, lys-382) of p53 at its C-terminal leading to activation of p53-DNA binding activity (Kouzarides 2000; Sterner and Berger 2000). Histone acetyltransferase (HAT) mediates the transcription of p53 (Brooks and Gu 2003). Both the covalent linkage of the acetyl group to lysine and the enzymatic reaction of acetylation were first identified on histones and its importance in transcriptional regulation was understood (Jenuwein and Allis 2001; Berger 2007). However, p53 was the first nonhistone protein to be activated by acetylation (Gu and Roeder 1997; Luo, Su et al. 2000). As a result of increasing cellular stress, the acetylation levels for p53 are significantly enhanced (Luo, Su et al. 2000; Barlev, Liu et al. 2001; Ito, Lai et al. 2001; Luo, Nikolaev et al. 2001; Vaziri, Dessain et al. 2001; Knights, Catania et al. 2006; Li, Piluso et al. 2007; Kim, Chen et al. 2008; Zhao, Kruse et al. 2008). With other words acetylation of p53 is a result of exposing the cell to cellular stress (Sakaguchi, Watanabe et al. 1998; Liu, Jin et al. 1999; Pearson, Carbone et al. 2000; Gottifredi and Prives 2001; Ito, Shinkai et al. 2001). In addition it was demonstrated by various groups (Sakaguchi, Sakamoto et al. 1997; Liu, Jin et al. 1999; Ito, Lai et al. 2001) that p53 can be acetylated by p300/CBP associated factor at lys-320. Acetylation of p53 is regulated by phosphorylation, which increases the interaction between p300/CBP and p53 (Lambert, Kashanchi et al. 1998; Dumaz and Meek 1999; Dornan and Hupp 2001). *In vivo* acetylation of K164 was demonstrated in p53 protein by PCAF and this acetylation was added to

the mode of action required to activate p53 (Tang, Zhao et al. 2008). All amino acids residues affected by acetylation are represented in **Figure 4**.

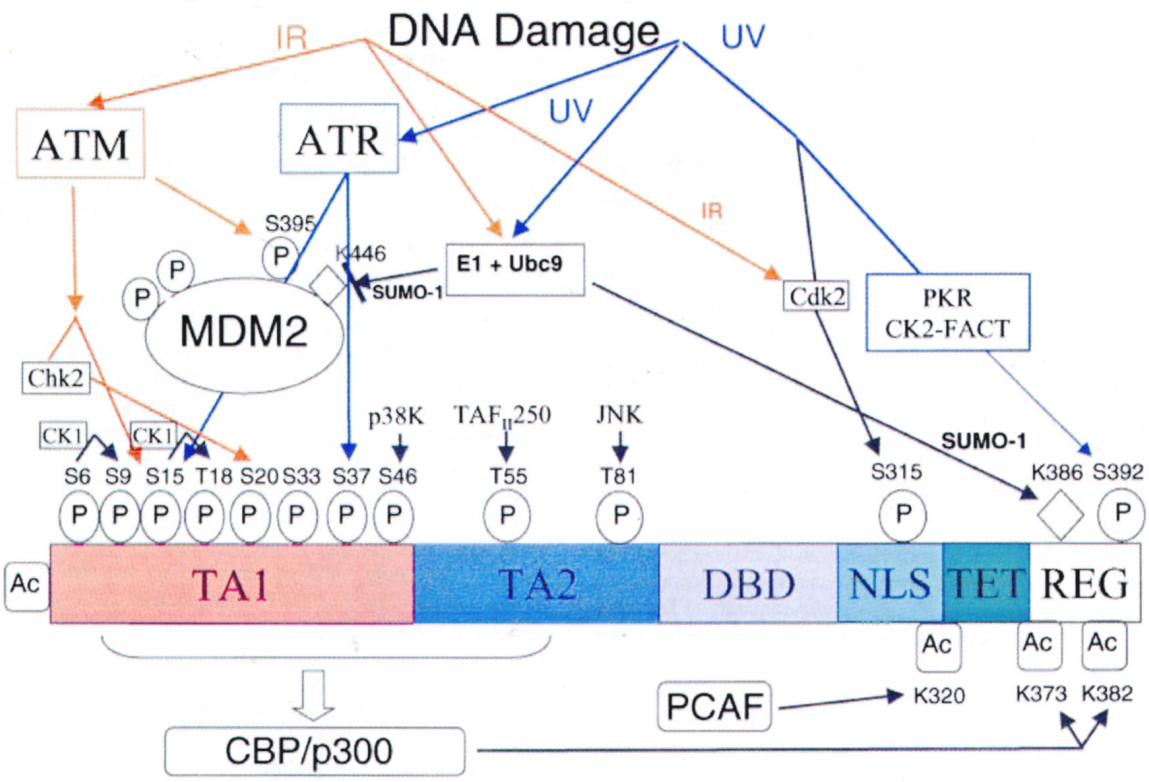


Figure 4
DNA damage mediates post-translational modification for p53
P53 is shown by a bar with 393 amino acids. P53 regions are transactivation domain (TA), DNA binding domain (DBD), nuclear localization (NLS), tetramerization domain (TET), and regulatory domain (REG). The phosphorylation (CP) and acetylation (AC) positions are represented by ovals and squares respectively. The numbers refer to the amino acids. Adapted from the review of Appella and Anderson 2001.

1.4 Functions of p53 protein

P53 acts as a transcription factor for many genes responsible for cell cycle arrest DNA repair and apoptosis (Bates and Vousden 1996). It was demonstrated that the *p53* gene is not essential for normal development and cell growth because the cells lacking the *p53* gene develop normally, although they are prone to cancer (Donehower, Harvey et al. 1992). It was reported that some mutant p53 can block the tumour suppression function of the wild type p53 in a dominant negative manner leading to inhibition of p53 binding to DNA (Miller, Aslo et al. 1990). Li-Fraumeni syndrome is a genetic syndrome, leading to initiation of cancer at the age of 30 and these families suffer from oesteosarcoma, breast cancer and brain tumours. Li-Fraumeni syndrome is frequently associated with a germ line mutation in the *p53* gene (Malkin, Li et al. 1990; Srivastava, Zou et al. 1990). It was demonstrated that such germ line mutations of the *p53* gene are missense or nonsense mutations (Frebourg, Barbier et al. 1995). There are two entities of Li-Fraumeni syndrome, the classical Li-Fraumeni (LFS) and the Li-Fraumeni-like syndrome (LFL). It was shown that in 70% of LFS cases and in 40% of LFL cases there is a germ line mutation in the *p53* gene (Tennant, French et al. 1995; Bachinski, Olufemi et al. 2005). Nullizygous $p53^{(-/-)}$ mice, exhibit a defect in DNA repair and apoptosis resulting in the progression of tumours for 30 % of homozygotes at the age of 4.5 months and death after 10 months of age (Perry, Piette et al. 1993). The tumours that developed in such animals were principally, lymphomas and sarcomas. $P53^{(+/-)}$ mice were extensively affected with urinary bladder tumourgenesis when compared with $p53^{(+/+)}$ (Ozaki, Sukata et al. 1998). P53 knockout mice are accompanied with increasing malignant progression of cancers (Kemp, Donehower et al. 1993), and

they were easily affected with genotoxic carcinogens (Malkin, Li et al. 1990; Tennant, French et al. 1995). It was demonstrated that there are some differences between Li-Fraumeni families and p53 $^{(+/-)}$ mice according to p53 germ line mutations, where in the Li-Fraumeni families point mutations cause missense and nonsense mutations leading to truncated proteins, while in p53 $^{(+/-)}$ mice there are deletions that lead to the absence of p53 protein. Also from tumour incidence, the Li-Fraumeni families show that 50% of the individuals develop cancer around the age of 30, while in p53 $^{(+/-)}$ mice, cancers developed by the age of 18 months in 50% of the individuals (depending on lifespan of the mouse) (Harvey, McArthur et al. 1993; Donehower, Harvey et al. 1995).

Figure 5 summarises the current understanding of activation and function of p53.

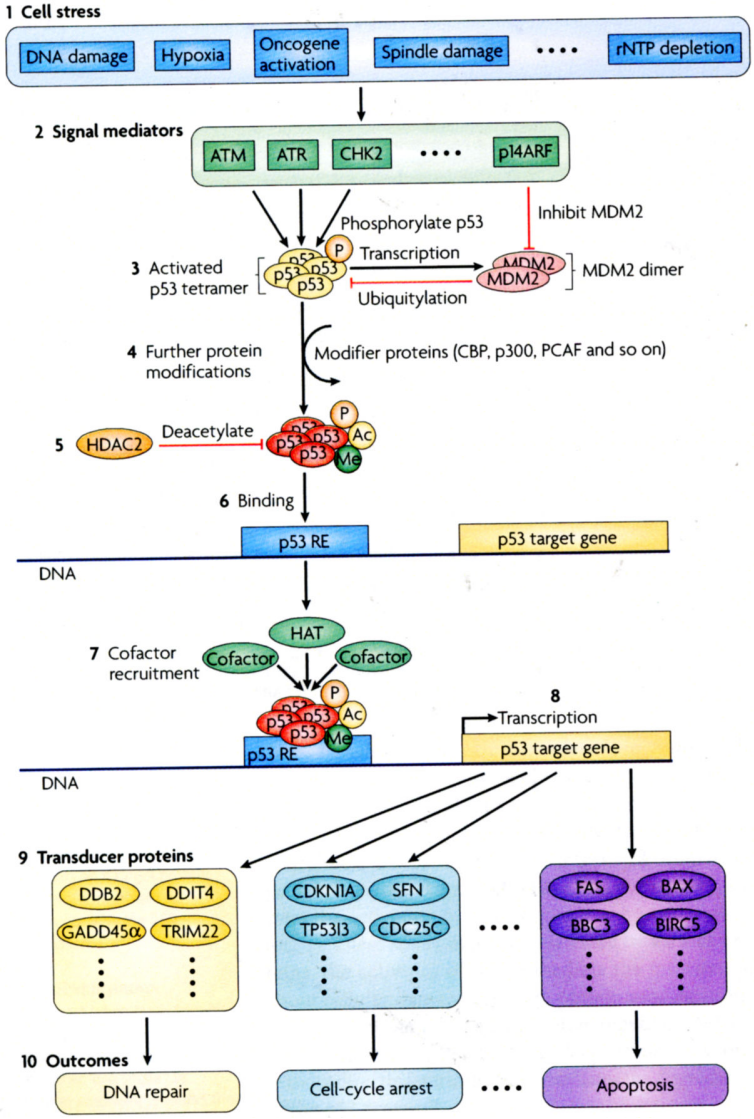


Figure 5
Activation and function of p53
Step 1: Cells are subject to many types of stresses most of the time. Step 2: Signal mediator proteins upregulate p53 by phosphorylation or suppressing ubiquitylation by MDM2. Step 3: Both processes increase the half-life of p53 which quickly leads to higher levels of p53. Step 4: Moreover p53 can be modified by acetyltransferases (CBP, p300, PCAF) and methyltransferases (SET9) that accumulate p53 protein and increase its DNA binding. Step 5: The deacetylase HDAC2 can inhibit p53 binding to DNA by deacetylation. Step 6: The p53 tetramer binds to a p53 response element (RE) to promote transcription of its gene. Step 7: P53 also needs cofactors such as histone acetyltransferases (HATs) and TATA-binding protein-associated factors (TAFs). Step 8: In this example, p53 mediates transactivation of its target genes. Step 9: P53 protein mediates various genes which produce proteins involved in various functions. Step 10: The previously activated proteins can perform DNA repair, cell-cycle arrest, senescence and apoptosis. ATM, ataxia telangiectasia mutated; BAX, BCL2-associated X protein; BBC3, BCL2-binding component-3; BIRC5, survivin; CDKN1A, cyclin-dependent kinase inhibitor-1A; CHK2, checkpoint kinase-2; DDB2, damage-specific DNA-binding protein-2; DDIT4, DNA-damage-inducible transcript-4; FAS, TNF receptor subfamily, member 6; GADD45α, growth arrest and DNA-damage inducible α; p14ARF; SFN, stratifin; TP53I3, tumour protein p53-inducible protein-3; TRIM22, tripartite motif containing-22. Adapted from Riley, Sontag et al. 2008.

1.4.1 p53 promotes cell cycle arrest

As a result of DNA damage, p53 is undergoning activation by ATM/ATR/CHK1/CHK2 and turning on the transcription of p21 (el-Deiry, Tokino et al. 1993; Kastan and Lim 2000; Bartek and Lukas 2003). Once p21 is activated, it binds to cyclin and cdk complex: cyclin D1-Cdk4, cyclin E-Cdk2, cyclin A-Cdk2 and cylin A-Cdc2 and causes cell cycle arrest at G1 phase. Moreover, it was demonstrated that p53 induces the cell cycle arrest at G2/M phase as well (Cross, Sanchez et al. 1995). Inhibition for cell cycle progression leads to stage of mitosis and it is induced after DNA damage or unrepaired damage originating from earlier S or G1 phase (Nyberg, Michelson et al. 2002; Xu, Kim et al. 2002). This role of p53 at G2 phase was confirmed when mitotic spindle inhibitors, such as noodazole was added to cells with wild type p53 protein. Surprisingly, the cell cycle was arrested at G2 phase while in case of absence of wild type p53 there was a progression of cell cycle and the DNA synthesis was reinitiated (Cross, Sanchez et al. 1995). The inhibition of cell cycle at G2 phase relies on the transcriptional proteins mediated by p53 such as p21, GADD45α (growth arrest and DNA-damage inducible 45 alpha) and 14-3-3 sigma protein (Taylor and Stark 2001; Nyberg, Michelson et al. 2002). From the previous results it is clear that p53 causes cell cycle arrest to keep the cell safe from accumulating any damage that happened to DNA. It was shown that p53 has the ability to stop the cell cycle at G0 phase by binding to *Gas1* gene (a membrane protein) and its function, once being activated, keeps cells in G0 phase for providing more protection to the cell (Del Sal, Ruaro et al. 1995). The scenario of the cell cycle is demonstrated in **Figure 6**.

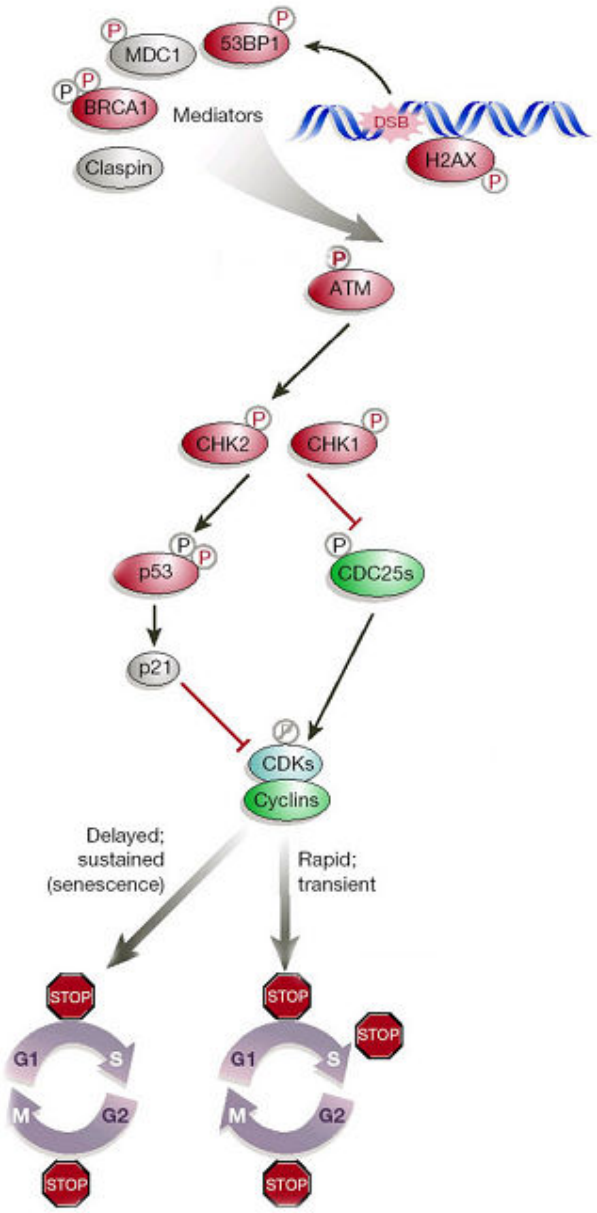


Figure 6
p53 mediates cell cycle arrest
One of the steps required for cell cycle arrest is DNA damage including double strand breaks (DSBs). Accordingly, some mediators (53BP1, BRCA1) activate ATM protein kinase. CHK1 and CHK2 mediate phosphorylation of p53 and inhibit CDC25s respectively. Once p53 is phosphorylated, it transactivates p21 which in turn inhibits CDKs (cyclin dependent kinases) with either delayed sustained or rapid transient speed depending on the degree of DNA damage. Adapted from Kastan and Bartek 2004.

1.4.2 Up-regulation of apoptosis by p53

As said before the tumour suppressor p53 protein plays an important part of the cell response due to DNA damage, either by inducing DNA repair or apoptosis. P53 inhibits the *bcl-2* gene which induces anti-apoptotic activity (White 1996). It was shown by many studies that p53 can mediate apoptosis by transactivation of series of pro-apoptotic proteins from the BCL2 family, including BAX, BID, PUMA and Noxa (Vousden and Lu 2002). These transactivated proteins induce mitochondrial membrane permeabilization (MMP) follwed by release of apoptotic factors from the mitochondrial intermembrane space. BAX protein has the ability to promote apoptosis due to the release of cytochrome c from mitochondria, which in turn activates the caspase cascade (Miyashita and Reed 1995). It was reported that p53 up regulates the protein ASK (activator of S-phase kinase) which mediates the activation of BAX and its interaction with mitochondria (Zhivotovsky and Kroemer 2004). P53 represses BCL2 (anti-apoptotic protein) which is expressed in mitochondria and inhibits membrane permeabilization (White 1996). It was shown that p53 due to DNA damage can stimulate the production of histone H1.2 promoting the induction of MMP (Konishi, Shimizu et al. 2003). It was observed that normal thymocytes undergo apoptosis after DNA damage, where the thymocytes from $p53^{(-/-)}$ mice do not undergo apoptosis when subjected to the same stimulus (Miyashita and Reed 1995). Moreover, p53 can also initiate apoptosis in a transcription independent way (Chipuk, Maurer et al. 2003). This mechanism is mediated by protein-protein interaction. P53 has the ability to bind to BCL2 and BCL-X_L which inhibits the function of both (Marchenko, Zaika et al. 2000; Mihara, Erster et al. 2003). Interestingly, apoptosis can be activated independently of p53. This can be achieved by involving p73, which acts as a p53-related transcription factor (Melino,

Bernassola et al. 2004). Apoptosis can be induced by NUR77 (also known as NGF1β or TR3) that can translocate to mitochondria and induce MMP after interacting with BCL2 (Lin, Kolluri et al. 2004). Therefore there might be a mechanism by caspase2 which can be activated in the nucleus by PIDDosome, a molecular complex that contains PIDD protein (p53-inducible death domain containing protein) and RAIDD, an adaptor protein containing a death domain (Tinel and Tschopp 2004). The pathway which represents p53 dependent and independent signals for apoptosis is shown in **Figure 7**.

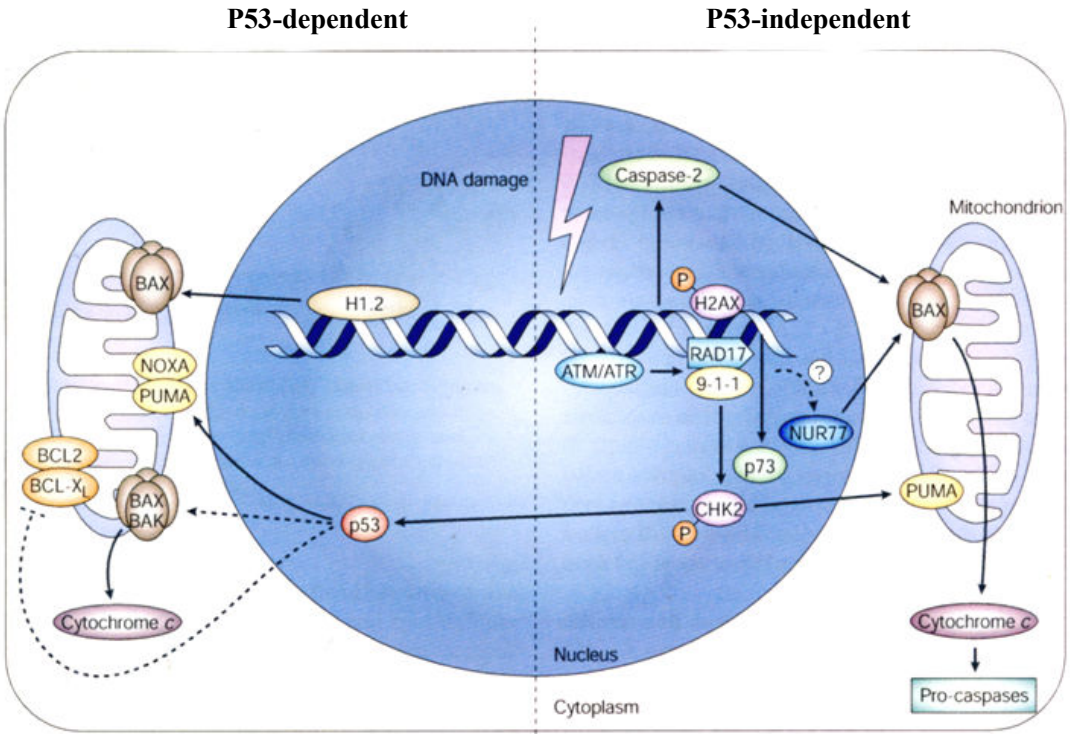


Figure 7
P53 dependent and independent apoptotic pathways
P53-mediated apoptosis might require the transcriptional activation of several genes that function at mitochondrial proteins (NOXA or PUMA) to induce apoptosis. P53 protein can promote a transcription-independent action, by direct interaction with BCL2 family, leading in turn to loss of anti-apoptotic activity of BCL2 or BCL-X_L while activating pro-apoptotic proteins i.e. BAX and BAK (dashed arrows). Moreover, damaged nuclei can release histone H1.2 in a p53-dependent way which works on mitochondria. Alternatively, DNA damage can mediate apoptosis in a p53-independent manner that involves caspase-2, NUR77 and p73. Taken together, it is clear that both p53-dependent and p53-independent apoptosis are present because the activation of the mitochondrial pathway is crucial for programmed cell death. ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; RAD17; histone H2AX; and CHK2, checkpoint kinase-2. Adapted from Zhivotovsky and Kroemer 2004.

Apoptosis can be induced by both an extrinsic and an intrinsic pathway (Fesik 2005). The extrinsic pathway (cell death signals) includes death receptors, for example FasL (Fas ligand) binding to Fas receptors. The Fas receptors bind to the DEAD domain (DD) resulting in FADD (Fas receptors dead domain) which in turn activates caspase 8 and the apoptotic pathway (Nagata 1997). It was demonstrated that the intrinsic pathway results from the interaction of cytochrome c with apoptotic protease-activating factor 1 (APAF1) and then binds to dATP forming a complex leading in

turn to activate caspase 9 which then activates the death response (Budihardjo, Oliver et al. 1999). Moreover, it was shown that p53 transports the FAS (death receptor protein) to the cell surface, consequently, apoptosis can be mediated (Bennett, Macdonald et al. 1998) **(Figure 8)**. The decision taken by the cell, for either growth arrest or apoptosis, is controlled by several factors including the cell type, the degree of DNA damage, survival factors and also environmental conditions (Chen, Chen et al. 1996) .

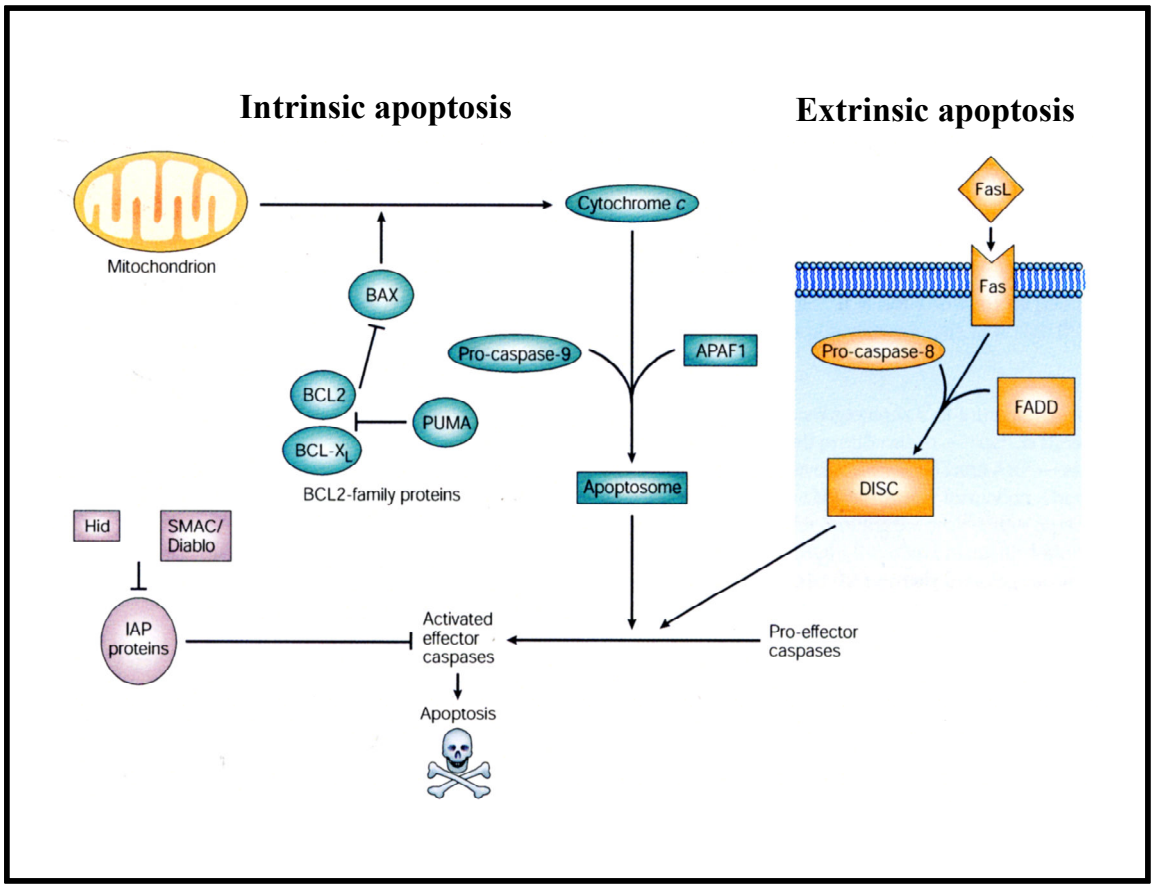


Figure 8
The intrinsic and extrinsic apoptotic pathways
The intrinsic death pathway starts with the release of cytochrome c from mitochondria in response to various stresses leading to the release of PUMA which, in turn, inhibits the pro-survival family members, BCL2 and BCL-X_L preventing binding to the pro-apoptotic protein, BAX. Since BAX mediates alteration of mitochondrial membrane permeability, cytochrome c is released. Cytochrome c promotes the formation of the apoptotic-protease-activating factor-1 (APAF1) and caspase-9-containing 'apoptosome' that initiates the caspase cascade, which in turn leads to cell death. The extrinsic pathway is activated by the interaction of secreted ligands, Fas ligand (FasL) to death receptors. Both of adaptor protein Fas-associated death-domain protein (FADD) and pro-caspase-8, are complexed within the death-inducing signalling complex, DISC. Cleavage and activation of caspase-8 in the complex promote the effector-caspase cascade, finally leading to cell death. Caspases are suppressed by inhibitor of apoptosis (IAP) proteins, which can bind to and block the active site on the caspase. IAPs are degraded by the activity of pro-apoptotic proteins like second mitochondria-derived activator of caspase (SMAC)/Diablo and its functional homologues in flies, which include Grim, Reaper and Hid. Adapted from Hipfner and Cohen 2004.

1.4.3 DNA repair function of p53

DNA repair mechanisms rely on many factors such as, DNA damage levels, the ability of cells for repairing and the time allowed for repairing (Gudkov and Komarova 2003). As said before, p53 is a multifunctional protein. It mediates the transcription processes for proteins involved in cell cycle arrest as well as DNA repair and apoptosis (Lane 1992; Prives, Bargonetti et al. 1994; Wang, Gibson et al. 1995). Using DNA-binding assays, it was shown that the affinity of p53 for mismatched and bulged DNA is equal to the human mismatch repair (MMR) complex, MSH2-MSH6, under the same binding conditions (Lee, Elenbaas et al. 1995).

In eukaryotes there are five main processes for DNA-repair, nuclear excision repair (NER), base excision repair (BER), MMR, non-homologous end-joining (NHEJ) and homologous recombination (HR) (Friedberg 2001; Tang and Chu 2002; Dianov, Sleeth et al. 2003; Lieber, Ma et al. 2003; Sancar, Lindsey-Boltz et al. 2004; Hoeijmakers 2007). Interestingly as shown in **Figure 9**, p53 promotes the DNA-repair process by both dependent and independent transactivation. This may explain, why p53 is acting as molecular node between the upstream signalling cascade and downstream DNA repair (Vogelstein, Lane et al. 2000).

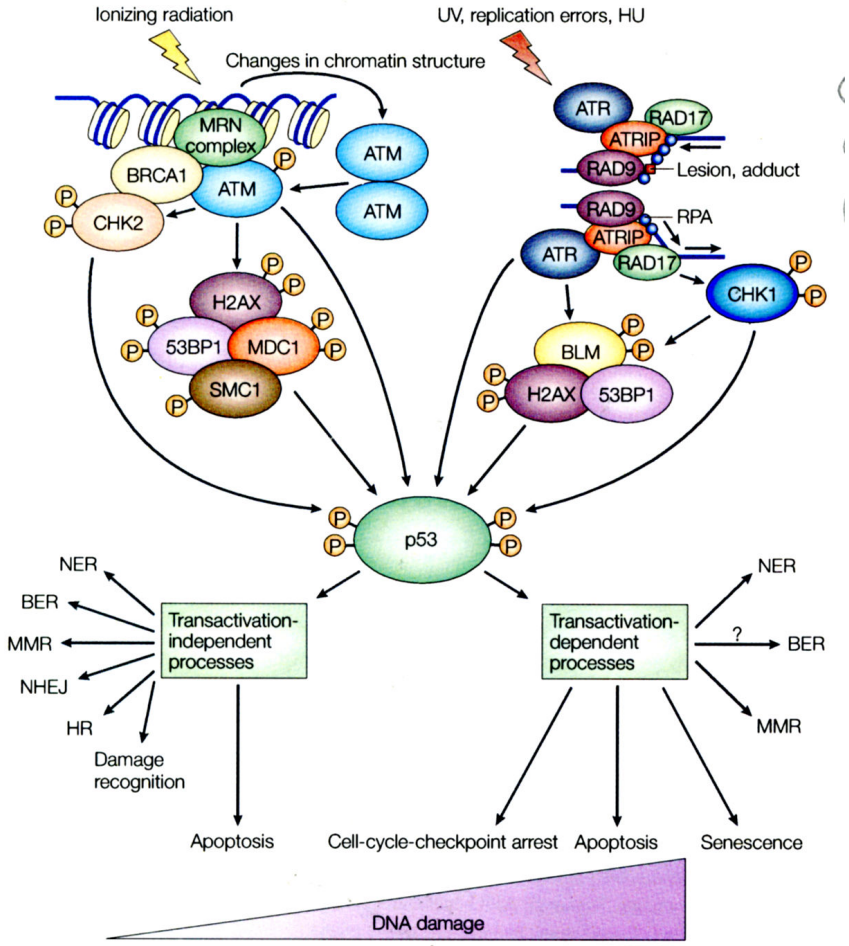


Figure 9
Functions of p53 in DNA repair
There are many causes for DNA damage such as ionizing radiation, UV, replication errors and HU. These events lead to the activation of ATM, ATR, CHK1 and CHK2 which in turn phosphorylate p53 protein at different amino acids in the sequence of p53. This activated p53 mediates many functions either through transactivation dependent or transactivation independent. The mediated process by p53 depends on the level of DNA damage and cell type. ATM, ataxia telangiectasia mutated; ATR-ATRIP, ataxia telangiectasia-and-RAD3-related-ATR-interacting protein complex; MRE11, mitotic-recombination-11; NBS1, Nijmegen breakage syndrome-1; BRCA1, breast cancer-susceptibility protein-1; H2AX, histone-2A family, member X; 53BD1, p53-binding protein-1; MDC1, mediator of DNA-damage checkpoint protein-1; SMC1, structural maintenance of chromosomes-1; RPA, replicating protein A; BLM, bloom syndrome protein; NER, nuclear excision repair; BER, base excision repair; MMR, mismatch repair; NHEJ, non-homologous end-joining; HR, homologous recombination; HU, hydroxyurea; UV, ultraviolet light. Adapted from Sengupta and Harris 2005.

It was shown that the loss of p53 in human cells leads to reduction in the repair process for damaged DNA (Smith, Chen et al. 1995; Wang, Yeh et al. 1995; Ford and Hanawalt 1997). Moreover, it was shown that p53 affects NER *in vivo* by regulating the transcription of proteins that are involved in this process including $p48^{DDB2}$ and XPC (Hwang, Ford et al. 1999; Adimoolam and Ford 2002). In this context it was demonstrated that p53 stimulates BER by interacting directly with APE1/REF1 and DNA polymerase β (Zhou, Ahn et al. 2001; Achanta and Huang 2004) **(Figure 10)**.

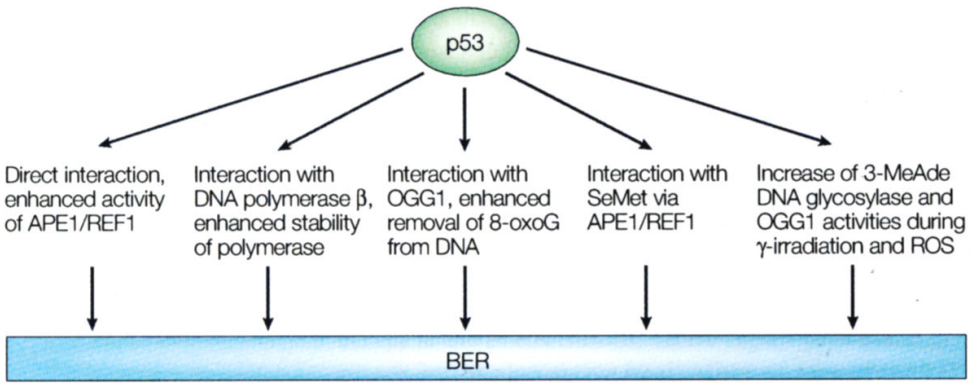


Figure 10
p53 and its role in base excision repair
P53 stimulates BER through binding with APE1/REF1, 8-oxoguanine (8-oxoG) and DNA polymerase-β. In the presence of ROS and γ-irradiation p53 transactivates 3-MEAde DNA glycosylase or OGG1 respectively. Adapted from Sengupta and Harris 2005.

MMR was shown also to be affected and regulated by p53 protein, where p53 is acting as a transactivator by binding to many response elements located in the promoter region of human MSH2 (Scherer, Maier et al. 2000). Surprisingly, *in vitro* p53-DNA-binding was enhanced by MSH2-MSH6 complex showing that MMR proteins and p53 protein work synergistically in cells (Subramanian and Griffith 2002). It was demonstrated that p53 binds to cellular proteins mediating DNA repair (e.g. replicating protein antigen, (RPA), xeroderma pigmentosum group B DNA helicase (XPB), xeroderma pigmentosum group D DNA helicase (XPD), P62, topoisomerase 1, and Cockayne's group B (CSB) (Dutta, Ruppert et al. 1993; Gobert, Bracco et al. 1996; Leveillard, Andera et al. 1996). Furthermore it was demonstrated that in the case of DNA damage by cellular stress (eg, UV light, X-rays, oxygen radicals (ROS)), p53 was expressed and mediates the expression of p21 which can cause cell cycle arrest at the G1 phase (Kuerbitz, Plunkett et al. 1992; el-Deiry, Tokino et al. 1993) and G2 phase (Hartwell 1992) to sustain the replication of DNA. Moreover, it was documented that if p53 failed to induce DNA repair during cell cycle arrest, it still has the ability to mediate apoptosis and inhibit the replication of the damaged DNA (Yonish-Rouach, Resnitzky et al. 1991; Hartwell and Kastan 1994). However, p53 can also mediate DNA repair by expressing p21 and GADD45. These two proteins can bind to PCNA which mediates DNA repair and replication (Flores-Rozas, Kelman et al. 1994). It was shown that GADD45 is a nuclear protein during cell cycle arrest after DNA damage and it activates PCNA function for DNA repair (Waga, Hannon et al. 1994). PCNA is capable of interacting with various proteins involved in DNA replication, repair, and translation and DNA synthesis supportting its role in regulating cellular DNA replication and repair processes (Warbrick 2000; Maga and Hubscher 2003; Prosperi 2006). Moreover, PCNA can

perform its role in DNA replication and repair through direct interacting with DNA polymerases inducing their processivity (Warbrick 2000; Maga and Hubscher 2003; Majka and Burgers 2004; Johnson and O'Donnell 2005; Prosperi 2006). PCNA can bind and stimulate AP endonuclease (e.g human APE2) (Tsuchimoto, Sakai et al. 2001). Interestingly, PCNA is required for early events of MMR (Umar, Buermeyer et al. 1996; Lee and Alani 2006) where it interacts directly with MSH6 and MSH3. P21 binds to PCNA and inhibits its function in DNA replication (Cahilly-Snyder, Yang-Feng et al. 1987; Flores-Rozas, Kelman et al. 1994). This inhibition mechanism was proceeded through displacement of DNA polymerases and other proteins that are involved in DNA replication (Gulbis, Kelman et al. 1996; Waga and Stillman 1998; Warbrick 2000; Cazzalini, Perucca et al. 2003; Perucca, Cazzalini et al. 2006).

1.5 The interaction between p53 and mdm2

1.5.1 A hint on mdm2

The *mdm2* (murine double minute) gene was detected in a spontaneously tumourigenic murine Balb/c 3T3 fibroblast cell line (3T3DM) in the molecular biology lab of Donna and co-workers (Cahilly-Snyder, Yang-Feng et al. 1987). It was shown that *mdm2* gene is localized on double minute chromosomes (Oliner, Kinzler et al. 1992). The full length human mdm2 protein contains 491 amino acids while the mouse mdm2 protein is 489 amino acids. This protein contains zinc fingers, an acidic domain and a putative nuclear localization signal (Fakharzadeh, Trusko et al. 1991; Oliner, Kinzler et al. 1992; Boddy, Freemont et al. 1994). The *mdm2* gene generates many mdm2 protein products. Some of these isoforms still have the ability to bind to p53 and others do not. The most frequent isoforms is $p85/90^{MDM2}$ which contains the entire structure for the main mdm2, $p74/76^{MDM2}$ and $p54/57^{MDM2}$ (Olson, Marechal et al. 1993; Haines, Landers et al. 1994; Gudas, Nguyen et al. 1995). It is noteworthy that the $p74/76^{MDM2}$ isoform can not bind to p53 protein because it lacks the N-terminal domain of the full length protein (Olson, Marechal et al. 1993; Haines, Landers et al. 1994). Moreover, it was shown that $p74/76^{MDM2}$ can antagonise the ability of $p85/90^{MDM2}$ to target p53 protein degradation (Perry, Mendrysa et al. 2000). By contrast the $p54/57^{MDM2}$ isoform can bind to p53 protein but it lacks the C-terminal domain (Olson, Marechal et al. 1993) **(Figure 11)**.

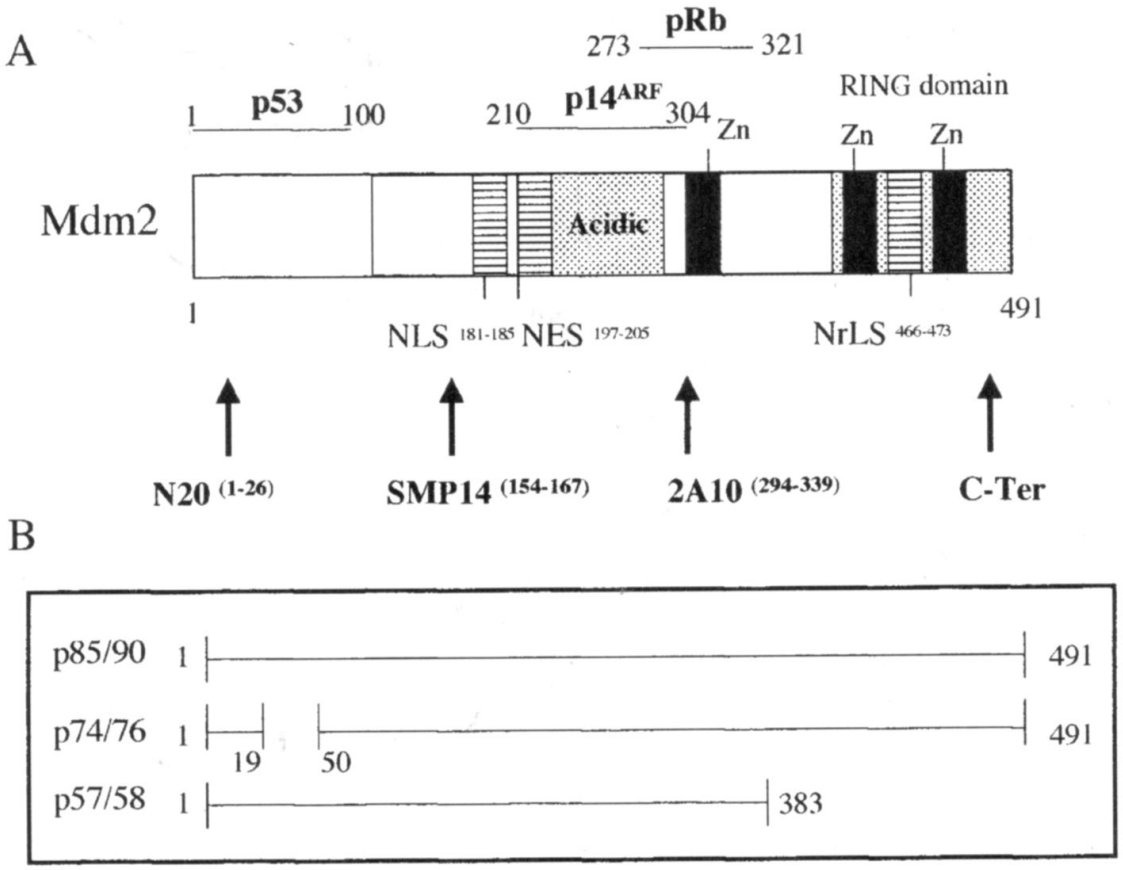


Figure 11
General structure of mdm2
(A) Different binding sites for p53, pRb and $p14^{ARF}$ with mdm2 protein at the top. Nuclear localization (NLS), export (NES) and signals (NrLS) are indicated by the hatched boxes. Zinc fingers are represented by black boxes. The full length protein contains a C-terminal and an acidic domain. The reactivity of the different antibodies (N20, SMP14, 2A10) with the mdm2 protein epitopes is demonstrated by↑.
(B) Comparison of the major mdm2 isoforms $p85/90^{MDM2}$ (contains all mdm2 epitopes), $p74/76^{MDM2}$ (lacking of N-terminal epitope between aa 19 to aa 50) and $p54/57^{MDM2}$ (deletion of C-terminal epitope). Adapted from Eymin, Gazzeri et al. 2002.

Brieflly, mdm2 is an oncoprotein targeting the degradation of p53 by acting as an E3 ubiquitin ligase (Oliner, Kinzler et al. 1992; Bueso-Ramos, Yang et al. 1993; Kubbutat, Jones et al. 1997; Fuchs, Adler et al. 1998; Ito, Lai et al. 2001). It was revealed that the *mdm2* gene was amplified in 33% of human sarcomas and 50% of human leukaemias (Oliner, Kinzler et al. 1992; Bueso-Ramos, Yang et al. 1993). As already described before, mdm2 plays an important role in the regulation of p53 (Marechal, Elenbaas et al. 1997) and loss of mdm2 leads to early embryonic lethality because of p53 de-regulation (de Rozieres, Maya et al. 2000). In response, mdm2 is also overproduced through transcriptional activation by p53. Hence, mdm2 acts as a negative feedback regulator for p53 (Oliner, Pietenpol et al. 1993; Wang, Michael et al. 2002; Bond, Hu et al. 2005). One of the consequences of mdm2 binding to p53 is inhibition of action as a transcription factor for many p53 dependent genes (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997; Wadgaonkar and Collins 1999) by targeting p53 for degradation through the ubiquitin-dependent proteosome pathway (Rovinski, Munroe et al. 1987; Hershko and Ciechanover 1998). Ubiquitin is a protein that consists of 67 amino acids and it binds to p53 at its N- terminal domain (Blaydes, Gire et al. 1997). There are several lines of evidence which revealed that mdm2 is the master regulator of p53 cDNAs which have a deletion mutation in the N- terminal domain of p53, yielding long half lives for p53 (Rovinski, Munroe et al. 1987). Moreover, it was shown that the level of p53 increases in cells treated with peptides that inhibit the formation of the mdm2-p53 complex (Blaydes, Gire et al. 1997; Kubbutat, Jones et al. 1997; Teoh, Urashima et al. 1997; Wasylyk, Salvi et al. 1999). Cells, which were treated with antisense constructs for blocking the expression of mdm2, produce high levels of p53 (Haupt, Maya et al. 1997) while cells, characterized by overproduction of mdm2, have a low level of p53 expression

(Haupt, Maya et al. 1997; Li, Brooks et al. 2003). The latter evidence demonstrates that cells which have the ability to produce an *mdm2* gene with a mutation disrupt the formation of the p53-mdm2 complex (Michael and Oren 2003).

1.5.2 Regulation of p53 activity by mdm2

It was reported that p53 faces two alternative fates, depending on mdm2 levels in the cell (Glickman and Ciechanover 2002):

1. In case of high levels of mdm2, p53 undergoes polyubiquitination and degradation within the cell nucleus.

2. Low levels of mdm2 drive p53 monoubiquitination and exportation from the nucleus. Therefore p53 protein is subject to polyubiquitination by the enzyme E4, followed by degradation in the cytoplasm via the 26S proteosome causing deubiquitination and transfer of p53 back into the nucleus for degradation by mdm2 together with nuclear E4 like proteins (e.g. p300) (Grossman, Deato et al. 2003). P53 which is exported to the cytoplasm could enter mitochondria and enhance the release of cytochrome c mediating apoptosis, **(Figure 12)**.

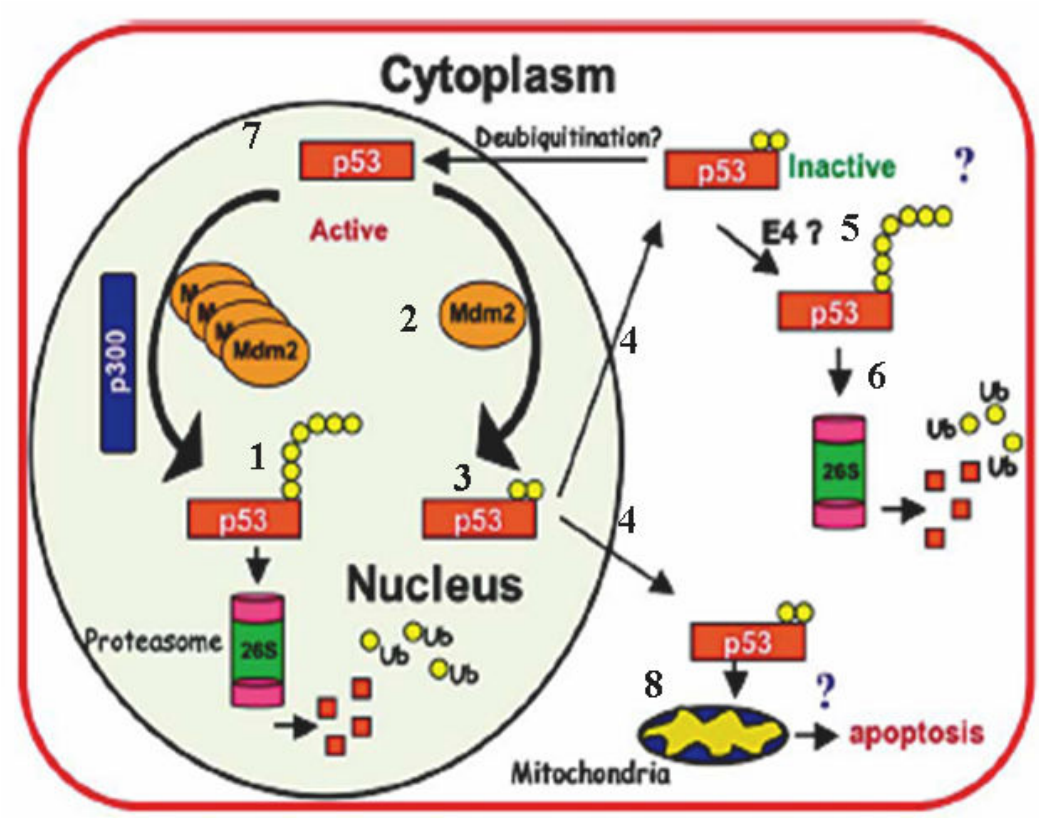


Figure 12
The relationship between p53 and mdm2
P53 undergoes polyubiquitination **(1)**, and degradation within the cell nucleus while **(2)** low levels of mdm2 drive p53 protein **(3)** monoubiquitination and **(4)** exportation from the nucleus into the cytosol, where p53 is subject to either **(5)** polyubiquitination by the enzyme E4 followed by **(6)** degradation on the 26S proteosome causing deubiquitination. P53 can be either **(7)** transferred back into the nucleus to be degraded by mdm2 together with nuclear E4 like proteins (e.g. p300), or **(8)** exported to enter mitochondria and enhance the release of cytochrome c mediating apoptosis. Adapted from Shmueli and Oren 2007.

Another pivotal p53 regulator via mdm2 protein is the E3 ubiquitin ligase which binds to p53 in the N-terminal domain promoting the covalent conjugation of ubiquitin residues leading to reorganisation by the 26S proteosome and ultimately to degradation of p53 (Michael and Oren 2003). Freedman and colleagues revealed that ubiquitin, which promotes p53 degradation by proteosomes, consists of multiple ubiquitin residues bound together covalently (Freedman, Wu et al. 1999). Inhibition of the mdm2 interaction with p53 in response to stress leads to the rapid stabilization

of p53 activating the p53 response (Ashcroft and Vousden 1999). Mdm2 function is also required for the efficient export of p53 from the nucleus to the cytoplasm (Giaccia and Kastan 1998). P53 is regulated primarily by its stability (Meek 1999). The most important signal for p53 stabilization and activation is DNA damage, which induces phosphorylation for the protein. This phosphorylation at ser15 affects its affinity for mdm2 and its subsequent degradation (Shieh, Ikeda et al. 1997; Lin and Lowe 2001; Shmueli and Oren 2007). Moreover, p53 stability, in response to oncogene signalling by increased levels of P14ARF (a tumour suppressor protein), can form a complex with mdm2, inhibiting its ability to degrade p53 (Braithwaite, Sturzbecher et al. 1987).

1.6 Protein nitration

Nitration is a chemical process where a phenol ring of L-tyrosine in proteins is subjected to nucleophilic attack by a nitrating agent such as NO_2. Protein nitration is considered now as post-translational modification (Mannick and Schonhoff 2004).

1.6.1 Formation and function of nitric oxide (NO)

In 1987, nitric oxide (NO) accounted for the bioactivity of endothelium–derived relaxing factor (EDRF) (Braithwaite, Sturzbecher et al. 1987). This led to widespread research on the physiological and pathological roles of this molecule. NO has important roles in vasodilatation, neurotransmission, inhibition of platelet aggregation and immune defence, and acts as an intracellular messenger for many cells (Ignarro, Buga et al. 1987). NO is characterized by a short half-life (3-30s), it is a colourless gas, soluble in water, and also highly soluble in organic solvents (Braithwaite, Sturzbecher et al. 1987; Nathan and Xie 1994). NO has an acidophilic nature giving it the ability to diffuse across cell membranes (Nathan and Xie 1994). NO is synthesised from the terminal guanido nitrogen atom of L-arginine by NADPH – dependent enzymes called NO synthases (NOS). There are three main isoforms of NOS. These are, neuronal (n) NOS, inducible (i) NOS and endothelial (e) NOS. nNOS and eNOS are expressed in neurones and endothelial cells, respectively. They can, however, be expressed by many other cells. Activation of the two isoforms depends on calcium ions and calmodulin resulting in production of NO at low concentrations (nmol) (Fukumura, Kashiwagi et al. 2006). iNOS requires induction by bacterial products or inflammation-associated cytokines produced in many cell types (eg macrophages) (Radi, Peluffo et al. 2001). The activation of the

enzyme iNOS leads to the production of high NO concentrations (10^{-6} M), without the presence of calcium ions for activation. There are several activities attributed to NO. The physiological effect is represented in binding to its guanylyl cyclase (GC) coupled receptors, leading in turn to conformational changes affecting GC activity and production of cGMP from GTP followed by modification in cell functions such as platelet disaggregation, synaptic plasticity and smooth muscle relaxation (Mannick and Schonhoff 2004). NO has the ability to modify proteins by a direct chemical reaction through S-nitrosylation which is the binding of NO to the thiolate group of a cysteine residues. This process modulates many protein functions (Stamler, Lamas et al. 2001). Another action for NO and its related species is found in oxidation and nitration of L-tyrosine in proteins (Mannick and Schonhoff 2004). NO-induced apoptosis is characterized by biochemical and morphological changes such as DNA and nuclear fragmentation, cell shrinkage, membrane blebbing and apoptotic body formation (Albina, Cui et al. 1993). The anti-tumor activity of NO is characterised by the inhibition of tumour cell proliferation, differentiation and metastatic spread. NO was shown to induce the expression of DNA-dependent protein kinase (DNA-PKcs), which are considered as pivotal enzymes in DNA repair (Xu, Liu et al. 2000). In the meantime, it was shown that the sensitivity of tumour cells to ionising radiation is determined by DNA-PK expression which confers protection against oxidative and nitrosative stress (Vaganay-Juery, Muller et al. 2000).

1.6.2 Formation of peroxynitrite

Peroxynitrite (PN) anion ($ONOO^-$), is generated by the rapid reaction of NO with $O_2^{\cdot-}$ (Burney, Caulfield et al. 1999). The rate of $ONOO^-$ formation depends on the concentration of $O_2^{\cdot-}$ and NO. Peroxynitrite attacks a wide range of biological molecules including lipids (Rodenas, Carbonell et al. 2000), DNA (Berlett, Friguet et al. 1996) and proteins (Radi 2004). The major result from the reaction of PN with proteins is the formation of meta nitro tyrosine. Thus, this product can be used as a marker for PN activity. It was shown that PN affects protein function, for example it can deactivate enzymes such as tyrosine hydroxylase (Messmer, Ankarcrona et al. 1994; Blanchard-Fillion, Souza et al. 2001) and prostacyclin synthetase (Szabo 2003). PN has been observed in many inflammatory conditions and it is a cytotoxic mediator. It is more active than $O_2^{\cdot-}$, H_2O_2 and NO alone (Messmer and Brune 1996).

1.6.3 The interaction between p53 and NO

It has been established that NO has effects on both cellular p53 levels and activity. Hence, there is a close relationship between both NO and p53 especially, since p53 is upregulated, when exposed to NO producing compounds or enzymes which might be due to DNA damage mediated by NO (Messmer, Ankarcrona et al. 1994; Forrester, Ambs et al. 1996; Calmels, Hainaut et al. 1997). P53 mediates transcriptional trans-repression of iNOS mRNA expression by a negative feed back loop (Calmels, Hainaut et al. 1997; Elias 2005) while mutant p53 does not perform this function. Also the exposure of p53 to a high level of NO leads to impairment of its DNA binding activity under *in vitro* conditions (Calmels, Hainaut et al. 1997). It was shown that, NO affects the expression and activity of oncogenes, which are

responsible for cell cycle and apoptosis (Messmer, Ankarcrona et al. 1994; Forrester, Ambs et al. 1996; Sandau, Pfeilschifter et al. 1997). These regulatory effects of oncogenes are specific for proper cell growth and differentiation. Both single (ss) and double (ds) DNA strand breaks caused by reactive nitrogen species (RNS) (Burney, Caulfield et al. 1999) have been reported (Merchant, Loney et al. 1996) but ss breaks are more common. It was demonstrated that DNA damage mostly occurs during replication and transcription where the tumor suppressor gene *p53* has the key role in repairing DNA (Moochhala and Rajnakova 1999). P53 blocks the cell cycle during G_1/S transition upon activation by radiation, DNA-damaging drugs, hyperproliferation, and hypoxia (Cadwell and Zambetti 2001) by expressing p21, a potent inhibitor of cyclin-dependent kinases (CDK). If DNA repairing mechanisms fail to correct genomic damage, apoptosis is initiated in the respective cells (Prives and Hall 1999). P53-induced apoptotic protein includes BAX (Miyashita, Krajewski et al. 1994; Miyashita 1997). At the same time wild-type p53 suppresses cellular survival factors such as BCL-2 (Miyashita, Harigai et al. 1994). Surprisingly, it was demonstrated that the most common mutations inactivating p53 are single amino acid substitutions, but preferable these mutations are favoured by sustained p53 expression due to genomic damage indirectly mediated by NO (Wink, Kasprzak et al. 1991).

1.7 The Human Epidermis

1.7.1 General structure of the epidermis

The skin represents the largest organ in the body. This organ is covering approximately 1.8m^2. It acts as biological barrier by separating internal homeostasis from the external environment. Moreover, it prevents the loss of water, is involved in excretion, regulates the temperature of the body, absorbs UV light and acts as an antimicrobial defence system (Holbrook and Wolff 1993).

The skin is divided into several layers, the epidermis and dermis.These layers are separated by the basement membrane. Interestingly, upper epidermis and dermis these two layers are not completely functionally separated, since they are participating together in numerous functions **(Figure 13)**.

1.7.2 The epidermis

The epidermis is characterized by being the outer most superficial layer of the skin. It is derived from the ectoderm and has the ability to be regenerated in approximately 30 days (Holbrook and Wolff 1993; Jensen, Lowell et al. 1999). Keratinocytes provide more than 90% of the cell populations. In addition there are dendritic melanocytes located on the basement membrane originating from the neural crest. Melanocytes play a crucial role in skin pigmentation. Moreover, there are other cells such as Langerhans cells and Merkel cells which are also dendritic cells. Stem cells have been found in the epidermis as well as along the basal lamina border (Lavker and Sun 1982; Christophers and Braver 1987).

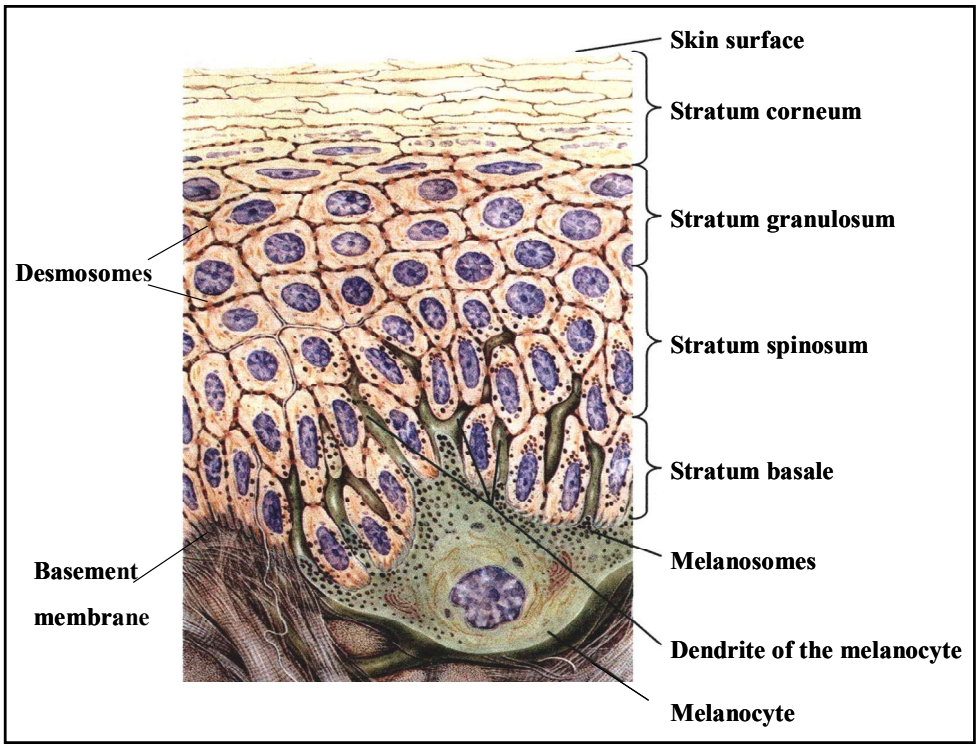


Figure 13.
Different cell types and layers of the human epidermis
Adapted from (Christophers and Braver 1987)

1.7.2.1 Keratinocytes

Keratinocytes originate from the ectoderm and they play a vital role in the barrier properties of the epidermis, in repair and regeneration. Keratinocytes are present in different layers of the epidermis forming the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum, the most superficial layer of the epidermis. This physiological turnover process takes approximately 30 days (Holbrook and Wolff 1993). The basal layer is characterized by active cell division. The basal keratinocyte is different from the other cells by possessing a columnar shape, containing a large nucleus and pigmented melanosomes transferred from surrounding melanocytes. The next layer in the epidermis is termed stratum spinosum referring to the presence of desmosomes which look like spines under the electron microscope. This layer occupies 3-4 layers in the epidermis with polyhedral shaped keratinocytes consisting of a rounded nucleus. The upper layer of the stratum spinosum contains large cells with a flattened nucleus. Moreover, these cells contain lipid rich organelles which are termed as lamellar granules. The important function of these lamellar granules is secreting their contents into the intracellular space, providing the stratum corneum with its characteristic barrier function properties (Feingold, Man et al. 1990; Elias and Feingold 1992; Holbrook and Wolff 1993; Feingold 2007; Sevilla, Nachat et al. 2007). Below the stratum corneum is the stratum granulosum. Here the cells have a flattened shape due to degradation including the loss of the nucleus and all cellular contents except the keratin filaments which mark the transition from the granular cell to the differentiated cornified cell within the stratum corneum (Holbrook and Wolff 1993). Undifferentiated keratinocytes induce melanocyte proliferation and melanogenesis, but also the differentiated keratinocytes stimulate melanogenesis utilising the symbiotic

relationship in the epidermal melanin unit (EMU) (Abdel-Naser 1999). This important role of keratinocytes is performed by releasing numerous factors such as pro-opiomelanocortin (POMC) derived peptides including α-melanocyte stimulating hormone (α-MSH), β-endorphin and ACTH/ACTH fragments which can stimulate the melanocyte in a paracrine fashion (Yaar and Gilchrest 1991; Kauser, Thody et al. 2004). Moreover, it was demonstrated that POMC-derived peptides and others including acetylcholine, catecholamines, nerve growth factor (NGF) and endothelin 1 are produced in an autocrine manner by the melanocyte itself (Cramer 1991; Imokawa, Yada et al. 1992; Imokawa, Yada et al. 1996; Gillbro, Marles et al. 2004; Kurzen and Schallreuter 2004; Hirobe 2005; Schallreuter, Kothari et al. 2008). By binding to their receptors located on melanocyte membrane, proliferation, differentiation, dendritogenesis and melanin synthesis of melanocytes can proceed (Sangiovanni 1819).

1.7.2.2 Melanocytes

Melanocytes are dendritic cells derived from melanoblasts which initially originated from the neural crest (Cramer 1991). Cutaneous melanocytes are sitting in the basal layer an average of 1000-2000 melanocytes/cm^2. Each melanocyte is accompanied by approximately 36-40 keratinocytes and this is described as the 'epidermal-melanin unit (Fitzpatrick and Breathnach 1963; Fitzpatrick, Miyamoto et al. 1967). The first description of pigment forming cells goes back to 1819 by Sangiovanni in 1819 who detected chromatophores in squid (Sangiovanni 1819). The first description of MC was in the human epidermis by Henle (Henle 1837). This close localization of both melanocytes and keratinocytes mediates symbiotic relationship between both cell types (Fitzpatrick and Breathnach 1963; Hadley and Quevedo

1966). For instance it was shown that keratinocytes can provide the noradrenergic melanocyte with adrenaline, while melanocytes transfer melanosomes to surrounding keratinocytes (Schallreuter, Wood et al. 1992; Holbrook and Wolff 1993; Spencer, Vestey et al. 1993).

1.7.2.3 Langerhans cells and Merkel cells

In addition to melanocytes and keratinocytes there are two other dendritic cells present. Langerhans cells originate from bone marrow and they present 2 to 8 % of the total epidermal cell population. The second cell type are Merkel cells. Langerhans cells are antigen presenting cells (Holbrook and Wolff 1993), while the function of the Merkel cells is still not completely clear.

1.7.3 Dermis

The dermis and subcutis represent the biggest percentage of the human skin. The dermis contains the fibrous and filamentous connective tissue. The main function of the dermis resides in providing the skin with the strength, elasticity and protection from mechanical injury. Moreover, the dermis provides the epidermis with nutrients and has receptors of sensory stimuli. The dermis is divided into two layers according to the differences in connective tissue structure, papillary and reticular regions. In the dermis collagen which is formed by mesenchymally derived cell is the major constituent and occupies 90%. The fibroblasts present in the dermis play a vital function in producing elastin providing skin with support and strength. Some other cells are located in the dermis such as macrophages, adipocytes, mast cells, lymphocytes and plasma cells along with appendages such as sweat glands, hair follicles and sebaceous glands (Holbrook and Wolff 1993).

1.8 The presence of nitric oxide in the skin

Endothelial NO is produced in the dermal vasculature by endothelial nitric oxide synthase (eNOS) and acts as a regulator for blood flow (Warren 1994; Feron 1999). The presence of inducible nitric oxide synthase (iNOS) has been shown in keratinocytes (Arany, Brysk et al. 1996), Langerhans cells (Qureshi, Hosoi et al. 1996), dermal fibroblasts (Wang, Ghahary et al. 1996), melanocytes (Rocha and Guillo 2001) and melanoma cells (Tsatmali, Manning et al. 1999) after stimulation with inflammatory cytokines and lipopolysaccharide (LPS). Keratinocytes contain both constitutive NOS (eNOS or NOS3 and nNOS or NOS1) which are activated by ultraviolet B (UVB) (Deliconstantinos, Villiotou et al. 1995; Villiotou and Deliconstantinos 1995). **Figure 14** shows the location of the different NOS in the human epidermis.

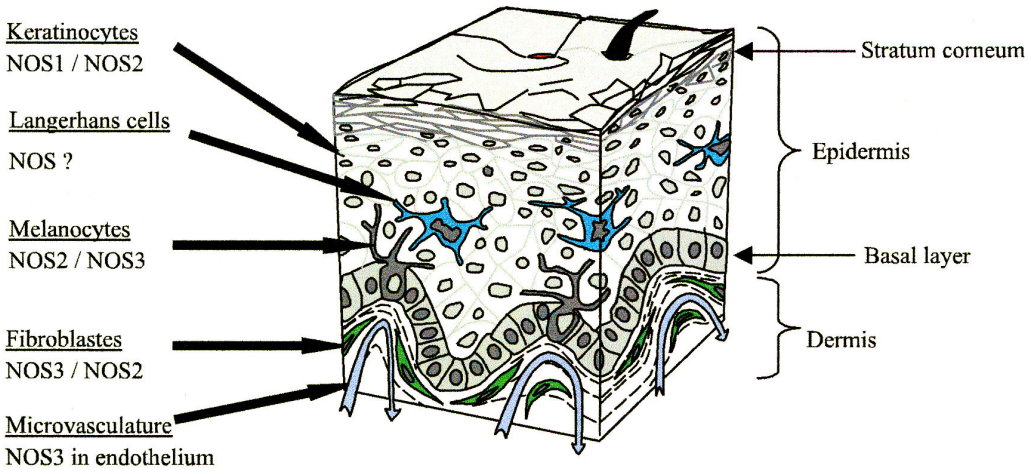


Figure 14
The formation of NO in the human epidermis
The expression of NOS isoforms in the skin. Adapted from Cals-Grierson and Ormerod 2004.

1.9 The formation of peroxynitrite in the skin

Nitrotyrosine is considered as a footprint for PN production (Beckman and Koppenol 1996; Crow and Ischiropoulos 1996). Nitrotyrosine has been detected *in vivo* by Western blotting or by ELISA in skin homogeneates during many conditions including chronic UVB exposure in rat skin treated with PN (Hattori, Nishigori et al. 1996) and moderate temperature burns (Rawlingson, Greenacre et al. 2000). NO and PN are produced in keratinocytes after ultraviolet B irradiation (Deliconstantinos, Villiotou et al. 1996; Deliconstantinos, Villiotou et al. 1996). After application of NO releasing cream to the skin, nitrotyrosine was formed and detected mainly in the epidermis revealing that exogenous NO may react with the skin $O_2^{\bullet-}$ to produce PN (Ormerod, Copeland et al. 1999).

1.10 Oxidative stress

Reactive oxygen species (ROS) are produced during many biological processes via biochemical reactions. ROS are neutralized by antioxidants but in some cases there is an imbalance between ROS and antioxidants leading to oxidative stress (Sies and Cadenas 1985). Important ROS molecules are hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$) (Poli, Leonarduzzi et al. 2004). These molecules are produced by many mechanisms in cells for instance exposure to X-ray, during drug metabolism and by cell metabolism where many redox reactions generate ROS (Schallreuter, Moore et al. 1999; Schallreuter, Moore et al. 2001; Schallreuter, Elwary et al. 2004; Schallreuter 2005; Schallreuter, Gibbons et al. 2006; Schallreuter, Gibbons et al. 2007). $OH^{\cdot}$ is the most reactive ROS due to its fast reactivity with DNA nucleotides leading in turn to DNA damage and oxidation of amino acids, finally causing protein malfunction and inhibition of enzyme activity. $OH^{\cdot}$ is produced from H_2O_2 in the presence of transition metals such as iron, copper etc. via the Fenton reaction or the Haber Weiss reaction (Haber and Weiss 1932; Goldstein and Czapski 1986) without involving transition metals.

$H_2O_2 + Fe^{+2} \longrightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$ Fenton Reaction

$H_2O_2 + O_2^{\cdot-} \longrightarrow OH^{\cdot} + OH^{-} + O_2$ Haber-Weiss Reaction

Nowdays it is clear that ROS are always present in the cell at low concentrations. It has been demonstrated that many signals are regulated by ROS (Halliwell and Gutteridge 1995; Vertuani, Angusti et al. 2004). However, ROS become dangerous to the cells with increasing levels due to post-translational consequences including oxidation and nitration. NO is a reactive nitrogen species and it has been involved in multiple pathological disorders including hypoxia and inflammation (Beckman and

Koppenol 1996). At the same time NO regulates various physiological processes, such as vascular and neurological functions (Fukumura, Kashiwagi et al. 2006). In the presence of both NO and $O_2^{\bullet-}$ in the cell, they yield the production of PN ($ONOO^-$). PN is a strong oxidizing agent, which has the ability to modify proteins via tyrosine nitration as well as oxidation (Cobbs, Brenman et al. 1995; Cobbs, Samanta et al. 2001).

1.11 Vitiligo-a model disease for oxidative stress

1.11.1 What is vitiligo?

Vitiligo is a disease affecting the entire epidermis where melanocytes lose their functionality, but both, keratinocytes and Langerhans cells, are also affected (Le Poole, van den Wijngaard et al. 1993; Ortonne and Bose 1993; Tobin, Swanson et al. 2000). The word vitiligo originates from vitula (latin) = calf and vitium (latin) = mistake. Vitiligo is characterised by white spots which can occur at any part of the skin and mucosa in different sizes. Based on the clinical picture vitiligo can be subdivided in different subgroups, vitiligo vulgaris, acrofacial vitiligo, segmental vitiligo and focal vitiligo (Schallreuter 2005; Schallreuter, Bahadoran et al. 2008). However, the reason for vitiligo is still unknown, but it can be said that it is a combination of many factors. Vitiligo affects 0.5-1% of the world population with no gender bias and age of onset starting from early childhood to old age (Ortonne and Bose 1993). By utilising WOOD's light (UVA 351nm), vitiligo can be early diagnosed due to fluorescence in the depigmented area based on the presence of oxidised pteridines (Schallreuter, Wood et al. 1994). Many mechanisms have been proposed for the aetiology of vitiligo. The main theory currently is still the autoimmune theory followed by the neural and the oxidative stress theory (Westerhof and d'Ischia 2007; Schallreuter, Bahadoran et al. 2008).

1.11.2. Evidence for H_2O_2 in the mM range in the epidermis of patients

The amount of H_2O_2 produced was measured in the depigmented epidermis by *in vivo* FT-Raman spectroscopy where its peak was characterised at 875cm^{-1} with levels in the mM range (Schallreuter, Moore et al. 1999). Nowadays multiple sources

within the epidermis have been identified which can contribute to the generation of this ROS (Schallreuter, Bahadoran et al. 2008). **Figure 15** summarise the current findings. These high concentration of H_2O_2 levels generated within the epidermis have many effects on the epidermal structure including DNA damage and oxidation of proteins and peptides (Spencer, Gibbons et al. 2007; Shalbaf, Gibbons et al. 2008; Schallreuter et al. 2008).

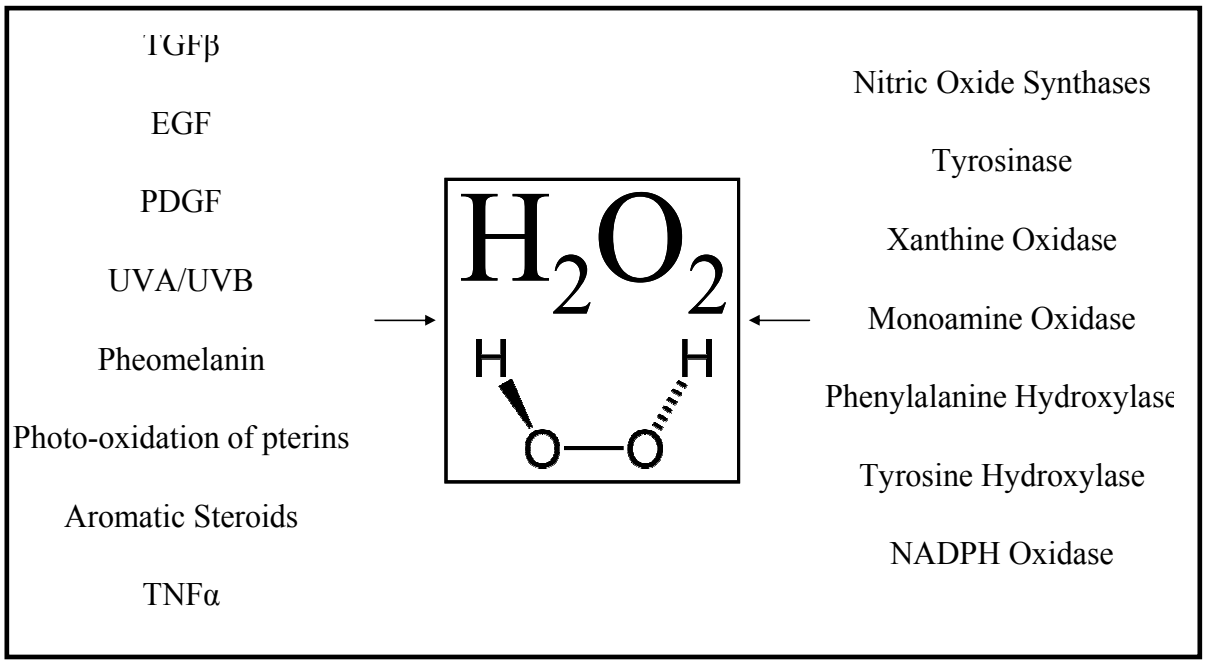


Figure 15.
Production of H_2O_2 in the human epidermis
H_2O_2 is produced by many ways such as UVA/UVB (Schallreuter and Wood 2001), photo-oxidation of pterin (Rokos, Beazley et al. 2002). H_2O_2 can also be produced by some enzymes, tyrosinase (Wood, Chavan et al. 2004), monoamine oxidase A (Barzu and Dansoreanu 1980; Maker, Weiss et al. 1981), xanthine oxidase (Olson, Ballow et al. 1974; Kellogg and Fridovich 1975), NADPH oxidase (Rossi, Della Bianca et al. 1985), tyrosine hydroxylases (Adams, Klaidman et al. 1997; Haavik, Almas et al. 1997) and the nitric oxide synthases as well (Parks and Granger 1986; Schallreuter, Wood et al. 1996). Moreover, it was shown that some factors are associated with the production of H_2O_2 such as transforming growth factor-β (TGF-β) (Shibanuma, Kuroki et al. 1991; Thannickal, Hassoun et al. 1993), platelet derived growth factor (PDGF) (Sundaresan, Yu et al. 1995), epidermal growth factor (EGF) (Bae, Kang et al. 1997) and TNFα (Hoffman and Weinberg 1987). In addition to the previous sources there are aromatic steroids such as oestrogen (Schallreuter, Chiuchiarelli et al. 2006) and progesterones (Martinoli, Zocchi et al. 1984). Pheomelanin has the ability to produce H_2O_2 in the fair skin due to its photolabile characters (Slominski, Tobin et al. 2004).

1.11.3 Detection of low epidermal catalase levels

Catalase enzyme plays an important role against ROS because it catalyses the reaction from H_2O_2 to O_2 and H_2O (Aronoff 1965). Catalase mRNA expression is unaltered in vitiligo as shown in epidermal suction blister tissue from lesional and non lesional epidermis of patients (Schallreuter, Moore et al. 1999). By contrast protein levels of catalase are significantly reduced in both lesional and non lesional skin (Schallreuter, Wood et al. 1991). Low catalase levels can be based on

degradation of its porphyrin active site by high concentration of H_2O_2 (Aronoff 1965) but also to oxidation of target amino acids in the enzyme active site and in the telomerisation domain (Gibbons, Wood et al. 2006; Maresca, Flori et al. 2006; Wood and Schallreuter 2006). Therefore a large variation in the loss of activity of catalase was detected in vitiligo ranging from 20-80% (Schallreuter, Wood et al. 1991). It was demonstrated that patients with vitiligo exhibit various types of polymorphisms in the catalase gene (Casp, She et al. 2002; Agrawal, Shajil et al. 2004; Gavalas, Akhtar et al. 2006; Park, Ha et al. 2006; Wood, Gibbons et al. 2008). In the epidermis of patients with vitiligo it has been established that activity of GTPCHI, the rate limiting enzyme for $6BH_4$ synthesis is increased 3-5 fold (Schallreuter, Wood et al. 1994). This scenario leads to an increase in $6BH_4$ production and increased $6BH_4$ levels have indeed been shown (Schallreuter, Wood et al. 1994). It is the oxidation of $6BH_4$ and other pterins via H_2O_2 that leads to the characteristic blue/green fluorescence under WOOD's light (351nm) seen in vitiligo (Schallreuter, Wood et al. 1994). Moreover, it should be noted that the oxidation product of $6BH_4$, 6-biopterin, is cytotoxic to melanocytes *in vitro* affecting both cell morphology and viability (Schallreuter, Büttner et al. 1994). Moreover, the final step of $6BH_4$ recycling via DHPR is also affected due to the oxidation of methionine residues leading to an altered NADH binding and a deactivated enzyme (Schallreuter, Moore et al. 2001; Hasse, Gibbons et al. 2004).

1.11.4 Constant up-regulation of epidermal wild type p53 in patients with vitiligo

Earlier our and other groups demonstrated that patients with vitiligo exhibit high protein expression of wild type functioning p53 in their epidermis. Protein levels

were not affected by removal/reduction of epidermal H_2O_2 via pseudocatalase PC-KUS. Therefore, it was suggested that H_2O_2 levels were still high enough even after reduction with pseudocatalase PC-KUS to induce transcription of p53 (Vile 1997; Schallreuter, Behrens-Williams et al. 2003). In this context it is interesting that H_2O_2 causes stabilization of p53 protein due to phosphorylation at ser 9, ser 15, ser 20 leading in turn to inhibition of mdm2 binding to p53 (Xie, Wang et al. 2001). It was demonstrated that ROS-mediated DNA damage induces the protein kinase ATM which phosphorylates p53 on ser 395 (Akman, O'Connor et al. 2000). P53 is considered to be the major effector of the genotoxic stress-signalling pathways induced by ATM (Caspari 2000). Recently it was revealed that p53 induces cutaneous pigmentation after UV irradiation by mediating the transcriptional activation of *POMC,* while in the absence of p53 the tanning response was inhibited (Cui, Widlund et al. 2007). Here it is of interest that POMC cleavage and the derived peptides are also targets for H_2O_2-mediated oxidation (Spencer, Chavan et al. 2005; Spencer, Gibbons et al. 2007). Moreover, it was demonstrated that p53 can play an important role in starting melanogenesis by mediating the transcription of HNF-1α which in turn controls MITF and tyrosinase (Schallreuter, Wazir et al. 2004). Interestingly, those results were confirmed after exposure of the skin to UV (Yang, Zhang et al. 2006). Moreover, it was shown that $O_2^{\cdot-}$ that originates from released oligonucleotides can produce H_2O_2 which in turn activates p53 (Atoyan, Sharov et al. 2007).

2.0 AIM

Nowadays there is accumulating evidence that the entire epidermis of patients with vitiligo exhibits oxidative stress based on mM (10^{-3}M) concentrations of H_2O_2 (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008). Moreover, Schallreuter and others showed the presence of up-regulated wild type p53 during apoptosis could not be detected in patients with vitiligo (Tobin, Swanson et al. 2000; van den Wijngaard, Aten et al. 2000; Schallreuter, Behrens-Williams et al. 2003). In addition, it was shown that NO affects both cellular p53 levels and activity, where p53 protein is up-regulated after exposure to NO leading in turn to DNA damage (Messmer, Ankarcrona et al. 1994; Forrester, Ambs et al. 1996; Messmer and Brune 1996; Calmels, Hainaut et al. 1997). To the best of our knowledge, the effect of both ROS and RNS in the human epidermis of patients with vitiligo has not been investigated so far. Therefore, many aims were of interst in this thesis.

1-What is the reason behind constant up-regulation of p53 in vitiligo?

2-How could ROS and RNA affect p53-DNA binding capacity?

3-Given the presence of severe oxidative stress in the epidermis of patients with vitiligo, oxidative DNA damage was highly expected. Therefore, we wanted to know which role p53 could play in this scenario.

3.0 MATERIALS AND METHODS

3.1 Cell culture

3.1.1 Epidermal melanocytes and keratinocytes

In order to establish epidermal melanocytes and keratinocytes cell cultures, a piece of skin was obtained from routine cosmetic enhancement. The fat layer was removed and the skin was washed in phosphate buffered saline (PBS) (Sigma, Dorset, UK). To facilitate the separation of the epidermis from dermis, some incisions were made in the upper layer. Thereafter the skin sample was incubated overnight in the fridge (4°C) in dispase (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, USA). On the next day the epidermis was separated from the dermis by peeling. Followed by a wash in 5% penicillin/streptomycin (Life Technologies, Paisley, UK) and 5% fungisone (Life Technologies, Paisley, UK) the sample was placed into sterile PBS. In order to isolate both melanocytes and keratinocytes from the epidermis, a cell suspension from the whole epidermis was made by incubating the isolated epidermis for 10 minutes at 37°C in 1 x trypsin/EDTA (Life Technologies, Paisley, UK). Thereafter, the medium154 containing keratinocytes specific growth factors, 0.1mM calcium chloride (Cascade Biologics, Mansfield, Nottinghamshire, UK) and 1% P/S was added to stop the reaction because long incubation with trypsin can damage the cells. The resulted suspension was centrifuged at 500 x g for 10 minutes at RT. The supernatant was discarded and the cell pellet containing both melanocytes and keratinocytes was resuspended in 10 ml of medium154 containing 1% P/S and transferred into 75cm^2 culture flasks (Scientific Laboratory Supplies, Nottingham, UK). For the nutritional requirements

of melanocytes and keratinocytes, the medium was changed regularly every two days.

3.1.2 Isolation of melanocytes and keratinocytes in cell cultures

The culture obtained is called "co-culture" because it contains both melanocytes and keratinocytes. To separate melanocytes from keratinocytes, the selective trypsinisation needs to take place. Melanocytes detach from the flask in approximately 1-2 minutes when 1 x trypsin/EDTA (Life Technologies, Paisley, UK) solution is added, whereas keratinocytes take at least 5 minutes. Detached melanocytes, were transferred into 75cm^2 culture flasks (Scientific Laboratory Supplies, Nottingham, UK) containing 10 ml of medium 254 supplemented with melanocyte specific growth factors and 1% P/S (Cascade Biologics, Mansfield, Nottinghamshire, UK). Keratinocytes were cultured in 10 ml of medium 154 (Cascade Biologics, Mansfield Nottinghamshire, UK) in a 75cm^2 culture flask.

3.1.3 Maintenance of cell cultures

The last step is allowing cells to attach and proliferate until 70-80% confluence is reached. For this reason cells were kept at 37^{o}C, 5% CO_2 and 95% humidity. Usually it takes 7-10 days to reach this level of confluence. During this period the medium is changed every 2 days. To increase the amount of cells, they were trypsinised and split into 75cm^2 flasks. Cells were cultured until reaching passage 3. Keratinocytes cell cultures were obtained as undifferentiated and differentiated cells. Differentiation was obtained by elevation of calcium chloride concentration to 2 mM.

3.1.4 Preparation of epidermal melanocyte and keratinocyte cell extracts

Both melanocyte and keratinocyte cell extracts were obtained by scraping the cells of the surface of the T75 flask into 10 ml of 1 x PBS. The cell suspension was centrifuged at 14,000 x g and resuspended in 200 µl of 0.05 M Tris pH 7.4 (Sigma, UK) followed by grounding with a pestle and mortar. The supernatant was aliquoted and stored at -80°C until further use.

3.2 Preparation of cell extracts from epidermal suction blister tissue

200 µl of 10 mM Tris buffer (pH 7.5) (Sigma, Dorset, UK) were added to the epidermal sheet. To get a good grinding, a very small amount of sand was added to the frozen skin. Another 200 µl of 10 mM Tris buffer (pH 7.5) was added under continuous grinding until no piece of epidermis could be seen. The liquid including sand was transferred to an Eppendorf tube and centrifuged at 14,000 x g for 10 minutes at RT in a bench top MSE MicroCentaur centrifuge (Sanyo, UK). The supernatant was removed carefully and aliquoted into separate Eppendorf tubes.

3.2.1 Protein determination

Total protein concentrations were determined by using the Bradford assay following the manufacture's protocol (BioRad Laboratories LTD., Hemel Hempstead, Herts, UK).

3.3 Immunohistological Methods

3.3.1 Cryosection preparation

Before cutting of the skin biopsy, slides holding the skin sections have to be coated with poly-L-lysine (Sigma, Poole, Dorset, UK) to allow the cryosections to adhere to the glass slide. The coating process was done by washing the slides in distilled water for 15 minutes, and then immersed in 70% alcohol for about 10 minutes, followed by coating for 10 minutes with 10% poly-L-lysine and finally air drying. A 3 mm punch biopsy was taken under local anaesthesia from the inner proximal arm as full thickness skin from consented volunteers, transferred into optimal cutting temperature compound (OCT) (Sakura, RA Lamb, Eastbourne, UK) for about 5 minutes to allow the skin to be frozen in liquid N2. 3-5 μm sections of this skin were cut using a Leica CM3050 S cryostat (Leica Microsystems, Milton Keynes, UK) and placed onto the poly-L-lysine coated slides. The slides were saved at -80°C for future work.

3.3.2 Preparation of chamber slides with cell cultures

To do *in vitro* immuno fluorescence studies, cells (melanocytes and keratinocytes) were cultured in chamber slides (Nalge Nunc International, Naperville, IL, USA). 100 μl cells (approximately 1 x 10^4 cells) and 300 μl medium were added to each chamber. After a few days each chamber was approximately 70-80% confluent. The medium was removed and cells were washed 3-4 times in sterile 1 x PBS, pH 7.4 followed by fixation in cold methanol (-20°C) for 10 minutes, then the slides were stored at -20°C until future work.

3.3.3 *In situ* immuno fluorescence labelling

Frozen slides were allowed to defrost at RT for 10 minutes followed by dehydration in 1 x PBS for 5 minutes. Fixation was performed by immersing slides for 6 minutes in ice cold- methanol. Except for mdm2 protein expression the slides were fixed in ice cold- acetone. Then slides were washed in 1 x PBS for 5 minutes, followed by blocking with normal donkey serum (10% NDS, Jackson Immunoresearch Laboratories, Cambridgeshire, UK) for 90 minutes at RT. Slides were washed in 1 x PBS for 5 minutes, followed by overnight incubation at 4°C with the primary antibodies, diluted in 10% NDS. In our experiment we used the following antibodies.

Antibody	Dilution	Incubation time/t	Source	Company
p53 (Ab-6)	Neat	Overnight, 4°C	Monoclonal mouse IgG2	Oncogene Research products, UK
p90MDM2	1:20	Overnight, 4°C	Mouse monoclonal IgG2a	Abcam, Cambridge, UK
p76MDM2 (SMP14)	1:20	Overnight, 4°C	Mouse monoclonal antibody	Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA
Inducible nitric oxide synthase (iNOS)	1:50	Overnight, 4°C	Polyclonal rabbit anti-human	Abcam, Cambridge, UK
Anti-nitrotyrosine	1:25	Overnight, 4°C	Polyclonal rabbit anti-human	Upstate, UK
p21	1:25	Overnight, 4°C	Mouse monoclonal antibody	Santa Cruz Biotechnology. UK
PCNA	1:50	Overnight, 4°C	Rabbit polyclonal antibody	Abcam, UK, Cambridge
ATM	1:50	Overnight, 4°C	Rabbit monoclonal antibody	Abcam, UK, Cambridge

Thereafter, slides were washed twice in 1 x PBS for 5 min followed by another wash once in Tween-20 (0.05%) for 5 minutes and then once again in 1 x PBS for 5

minutes. After that, slides were incubated for 1 hour at RT with a fluorescent secondary antibody in a dilution of 1:100 in 1% NDS for 1 hour (donkey anti-rabbit, rat, mouse or sheep depending on the primary antibody that was used) (Jackson Immuno Research Laboratories, Cambridgeshire, UK). Slides were washed twice in 1 x PBS for 5 minutes, then once in Tween-20 for 5 minutes followed by a final wash in 1 x PBS for 5 minutes. Finally, slides were dried and mounted using Vectashield Mounting Medium containing 4, 6-diamino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) and covered with a coverslip. Viewing the pictures was carried out by using a Leica DMIRB/E fluorescence microscope (Wetzlar, Germany). Images were captured using a Nikon Eclipse 80i microscope with a DS-U101 Nikon camera coupled to a Nikon ACT-2U capture programme (Nikon, Europe). *In situ* double immunofluorescence was performed in cryosections in order to detect two specific antibodies within one section. After incubation of the sections with secondary antibody it was blocked again as described before, followed by incubation with the second primary antibody after that the previous procedures were followed. Paint Shop Pro™ 7 was used to merge the two used different fluorochromes, tetramethyl rhodamine isothiocyanate (TRITC) and flluorescein isothiocyanate (FITC) (Jackson Immuno Research Laboratories, Cambridgeshire, UK).

Control staining was carried out by removing the primary antibody from the staining procedure and substitution of 1% NDS / PBS.

3.3.4 *In vitro* immuno fluorescence labeling

The staining procedures were carried out as described under *in situ* immuno-reactivity studies. The same antibodies with the same dilutions were utilised.

However, washing times were decreased because prolonged washing could damage the cells.

3.3.5 Quantification of fluorescence intensity and statistical analysis

To quantify the fluorescence, ImageJ version 1.37 was utilised (supplied online by NIH at http://rsb.info.nih.gov/ij/index.html). The region to be quantified was highlighted and a mean intensity obtained. This was repeated for the rest of the layers. The procedure was repeated three times for the region and the mean value was obtained and plotted. Statistical analysis was performed by Graph pad prism version 4 and 1-way ANOVA with Bonferroni analysis. The mean of the calculated values were plotted in the figures.

3.4 SDS-PAGE of protein samples

Electrophoresis analysis of protein samples was performed by using SDS-polyacrylamide gel consisting of a 10% w/v resolving gel (3.8 ml of distilled water, 3.4 ml of 30% acrylamide mix, 2.6 ml of 1.5 M Tris pH (8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium persulphate, 0.004 ml of TEMED) (Sigma, Dorset, UK) and 5% w/v stacking gel (4.2 ml of distilled water, 1 ml of 30% acrylamide mix, 0.76 ml of 1.5 M tris pH (8.8), 0.06 ml of 10% SDS, 0.06 ml of 10% ammonium persulphate, 0.006 ml of TEMED) (Sigma, Dorset, UK). Samples were transferred from -20°C and thawed at RT and loaded together with a protein ladder (#7720, Cell Signalling, UK). The gel was run at 90 volts for about 2 hours in Tris-Glycine buffer (25 mM Tris, 250 mM glycine, 0.1% w/v SDS) at pH 8.3. Afterwards the gel was immersed in Coomassie blue staining solution for about 5 min (50% v/v methanol, 10% v/v acetic acid and 0.2% w/v Coomassie Brilliant Blue R-250) (Sigma, Dorset, UK). In order to visualise any proteins, the gel was destained in solution I (50% v/v methanol, 10% v/v acetic acid in distilled water) for about 5 minutes, and finally immersed in the destaining solution Π (7.5% v/v methanol, 10% v/v acetic acid in distilled water) for 5 minutes to remove the excess dye.

3.4.1 Western blotting

In order to perform Western blotting, the protein samples were first resolved in SDS PAGE gel electrophoresis as described before. The gel was placed against a PVDF membrane (Millipore, MA, USA) sandwiched with filter paper solidly supported. The electro blotting chamber was filled with transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% w/v SDS and 20% v/v methanol) (Sigma, Dorset, UK). The

transfer was run at 20 volts for about 2hours. Afterwards the PVDF membrane was immersed in a blocking solution consisting of 5% w/v non-fat dry milk powder in 1 x PBS, 0.2% v/v Tween 20 and shaken for 1 hour at room temperature to block non specific binding sites. Then the membrane was washed 4 times for 1 hour in 1 x PBS and 0.2% v/v Tween 20 under continuously shaking. The membrane was incubated for 1 hour with the following corresponding antibodies to detect proteins.

Antibody	**Dilution**	**Incubation time/t**	**Source**	**Company**
p53 (DO-1)	1:1000	1 hour/RT	Monoclonal mouse IgG2	Novacastra, UK
p76MDM2 (SMP14)	1:1000	1 hour/RT	Mouse monoclonal antibody	Santa Cruz Biotechnology, Inc. Santa Cruz, CA, U.S.A
Inducible nitric oxide synthase (iNOS)	1:1000	1 hour/RT	Polyclonal rabbit anti-human	Abcam, Cambridge, UK
Anti-nitrotyrosine	1:1000	1 hour/RT	Polyclonal rabbit anti-human	Upstate, UK
p21	1:500	1 hour/RT	Mouse monoclonal antibody	Santa Cruz Biotechnology. UK
PCNA	1:1000	1 hour/RT	Rabbit polyclonal antibody	Abcam, UK
ATM	1:500	1 hour/RT	Rabbit monoclonal antibody	Abcam, UK

All the previous antibodies were diluted in 5% w/v non fat dry milk powder in 1 x PBS, 0.2% v/v Tween 20. This was followed by 4 washing steps within 1 hour in 1 x PBS under continuous shaking. The membrane was transferred into a plastic dish containing either a rabbit anti-mouse IgG antibody (Dakocytomation, Glostrup, Denmark, 1:1000) or anti-rabbit antibody HRP (Cell signalling, UK, 1:10000) in 5% w/v non fat dry milk powder in 1 x PBS, 0.2% v/v Tween 20 depending on the host

of the primary antibody and incubated for 1 hour at RT, followed by wash in 1 x PBS. Positive immunoreactivity was detected by the enhanced chemiluminescence's method (ECL). To do so, the membrane was immersed for 2 minutes in equal volumes of solution I (1 ml of 250 mM luminol in DMSO, 0.44 ml of 90 mM p-coumaric acid in DMSO, 10 ml of 1 M Tris-HCl pH 8.5 in a final volume of 100 ml) (Sigma, UK) and solution II (64 µl of 30% v/v H_2O_2, 10 ml of Tris-HCl pH 8.5 in a final volume of 100 ml) (Sigma, UK). Positively stained protein bands send a luminescent signal that was visualised on a sheet of X-ray film (Sigma, Dorset, UK).

3.4.2 Statistical analysis for Western blot

The bands were evaluated by utilizing Image J version 1.37 (supplied online by NIH at http://rsb.info.nih.gov/ij/index.html). Each band was highlighted and the intensity was calculated. Statistical analysis was performed by Graph pad prism version 4 and 1-way ANOVA with Bonferroni analysis. The mean of the calculated values were plotted in the figures.

3.5 Dot Blotting

Dot blot is a technique which mimics Western blot. This technique is used for identification, detection, and analysing of the required proteins. The mechanism of this method is to spot the protein samples directly onto the membrane instead of performing separation by electrophoresis.

3.5.1 Dot blotting of resolubilised p53 protein from *E.Coli*

Blot analysis was utilised to follow the effect of H_2O_2 on p53 protein. Oxidation of p53 (0.2 mg/ml) was performed with different concentrations of H_2O_2 (30%, Fluka, UK).

P53 protein was incubated with different concentrations of H_2O_2 (1, 5, 10, 50, 100 mM) for 1 hour at RT. Then, dry paper towel was placed on the surface. Next four filter papers previously immersed in 0.05% Tween 20 in TBS were put on top of these paper towels followed by a PVDF membrane (Millipore, MA, USA) prior soaked in methanol for 20-30 sec, followed by washing with distilled H_2O and finally transferred to 0.05% Tween 20 in TBS for 2-3 minutes. Protein samples were spotted directly onto the PVDF membrane and left to air dry (5-10 minutes). The blocking procedure was carried out by incubating the membrane with 5% w/v non-fat dry milk powder in 1 x PBS, 0.2% v/v Tween 20 for 2 hours at RT under shaking conditions. The next procedures included washing and exposing to primary and secondary antibodies until the developing process was carried out in the same way as Western blotting described in detail before.

3.6 Microbiological medium: Lauria-Bertani (LB)

This media consisted of 10 g of bacto-tryptone (Oxoid, Basingstoke, Hampshire, England), 5 g of bacto-yeast extract, 10 g of NaCl and was adjusted to pH 7.0 using 5 M NaOH, and then made up to a volume of 1 litre using distilled water. The medium was autoclaved at 120°C for 20 minutes. LB agar medium was prepared by adding 15 g of agar to 1 litre of LB medium before autoclaving. Where stated, LB medium was supplemented with ampicillin to a final concentration of 10 µg/ml.

3.6.1 Purification of plasmid DNA

Plasmid DNA was purified utilising the alkaline lysis method of Birnboim and Doly (1979) and Ish-Horowiz and Burke (1981) by using plasmid DNA isolation kits (Qiagen, England). 1.5 ml of overnight culture was placed into an Eppendorf tube and then centrifuged at 14,000 x g for 1 minute at RT in a bench top MSE MicroCentaur centrifuge (Sanyo, UK). The supernatant was discarded and the cell pellets were resuspended in 250 µl of buffer P1 (100 µg/ml RNase, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0). After 5 minutes 250 µl of P2 lysis solution (200 mM NaOH, 1% w/v SDS) was added and mixed by gentle inversion for a further 5 minutes to lyse the cells. Then 350 µl of neutralisation buffer N3 (5.6 M KAc, pH 4.8) was added to obtain a cloudy precipitate of denatured chromosomal DNA and proteins. After spinning the tube in a microcentrifuge for 10 minutes at 14,000 x g the supernatant was transferred to a separate QIAprep spin column and centrifuged at 14,000 x g for 30-60 sec at RT again in a microcentrifuge. The QIAprep spin column was washed with 0.75 ml buffer PE (1.0 M NaCl, 50 mM. MOPS, 15% v/v ethanol, pH 7.0) and centrifuged for 30-60 sec. The flow-through was discarded and the spin

column again was centrifuged at 14,000 x g for 1 minute to remove residual washing buffer. Then the QIAprep column was transferred to a sterile Eppendorf tube and 50 µl of elution buffer EB (10 mM Tris-HCl, pH 8.5) was added to the centre of QIAprep column to elute the plasmid DNA. After leaving the column to stand for 1 minute, plasmid DNA was recovered from the column by centrifuging at 14,000 x g for 1 minute in a microcentrifuge at RT. Plasmid DNA was stored at -20°C until further use.

3.6.2 Transformation of a BL 21/DE3 strain with pT7.7 Hup53 plasmid DNA using CaCl2

0.1 ml of a BL21/DE3 overnight culture was added to 10 ml of LB media in a 50 ml Falcon tube, and left to grow at 37°C while shaking at 180 rpm in a Gallenkamp orbital incubator. The OD_{650nm} was measured until the growth reached mid-log phase (OD_{650nm} 0.3-0.4) and then the culture was placed on ice for 10 minutes. 1.5 ml of culture was added to each of two 1.5 ml sterile Eppendorf tubes and centrifuged in an MSE MicroCentaur microcentrifuge at 14,000 x g for 1 minute at RT. The pellets were resuspended in 0.4 ml of ice-cold 0.1 M $CaCl_2$ solution (Sigma, UK) and left for 15 minutes before centrifugation at 14,000 x g for 1 minute. After discarding the supernatant the pellets were resuspended in 0.2 ml of ice-cold 0.1 M $CaCl_2$ solution and the competent cells aliquoted into two sterile Eppendorf tubes. 5 µl of plasmid pT7.7 Hup53 DNA (~0.1 µg) was added to one tube, while to the other (control) 5 µl of sterile distilled water was added. Both tubes were placed on ice for 20 minutes. Plasmid DNA uptake was promoted by transferring the tubes to a water bath at 45°C for 90 sec, and then transferred directly on ice for 5 minutes, to complete the heat shock step. 0.6 ml of LB medium was then added to each tube and was incubated and

shaking for 1 hour at 180 rpm. After 1 hour, the two sterile Eppendorf tubes were centrifuged in a microcentrifuge at 14,000 x g for 1 minute at RT. The pellets in the two sterile Eppendorf tubes were resuspended in 0.2 ml of LB broth, before being plated out onto dried LB agar plates supplemented with 10 µg/ml ampicillin followed by incubation at 37°C overnight.

3.6.3 Pilot experiment for determination of the optimal p53 expression time from the BL21/DE3 strain containing pT7.7 Hup53 construct (Midgley et al., 1992)

Two 50 ml Falcon tubes containing 10 ml of LB broth were supplemented with 100 µl of a BL21/DE3 pT7.7 HuP53 overnight culture and 100 µl of ampicillin (10 µg/ml). Another two 50 ml Falcon tubes containing 10 ml LB broth were also supplemented with 100 µl of BL21/DE3 overnight culture. All four cultures were grown in Falcon tubes at 37°C with shaking at 180 rpm in a Gallenkamp orbital incubator, until reaching the early log phase (OD_{650nm} 0.15-0.2). After 90 minutes, an aliquote of 800 µl was taken to measure the OD_{650nm}. Isopropylthiogalactopyranoside (IPTG) at a final concentration of 1 mM was added once the OD_{650nm} reached 0.15-0.2, which indicates the early log phase. The remaining cultures without IPTG supplement served as negative controls. The transferred 800 µl of the cultures was centrifuged in MSE MicroCentaur microcentrifuge at 14,000 x g for 1 minute at RT. Then the supernatant was discarded and the pellets were resuspended in 30 µl of 2 x SDS gel–loading dye (100 mM Tris-HCl pH 6.8, 10% w/v bromophenol blue, 20% v/v glycerol) and 6 µl of 100 mM DDT (Sigma, Dorset, UK) and boiled for 5 minutes. These and later samples were then stored at -20°C for further use. The culture was left to grow for 4 hours and after each hour the OD_{650nm} was measured by

removing 800 µl of the cultures from each tube, which were then processed for SDS-PAGE gel analysis.

3.6.4 Large scale expression and resolubilisation of human p53 protein

20 ml of overnight culture of BL21/DE3 pT7.7 HuP53 and 200 µl of ampicillin (10 μg/ml) was added to 2 litres of LB broth. The culture was kept in the Gallenkamp orbital incubator at 37°C and 180 rpm until the OD_{650nm} reached 0.15-0.2 (early log phase). At this stage IPTG was added to a final concentration of 1mM and the culture was left to grow for 4 hours (optimum condition). 4 hours later the culture was transferred in clear tubes and centrifuged (Beckmann, Avanti^{TM} J-25) at 16,000 x g for 20 minutes at 4°C using the large rotor (JLA, 10,500). The resulting pellets were resuspended in 20 ml cold 50 mM Tris-HCl (pH 8.0) and for preservation purposes 0.66 ml per gram cells of buffer (10% w/v sucrose, 50 mM Tris-HCl pH 8.0) was added prior to storage at -80°C. When required cells were thawed at 25°C and 15 µl of 10 mg/ml lysozyme and 20 µl of 5 M NaCl per ml of cells were added. Cells were kept for 45 minutes on ice and continuously mixed to allow complete cell lysis. Because the solution of lysed cells became very viscous, it was warmed in a water bath at 37°C for 1 minute, incubated again in ice water and sonicated three times at maximum amplitude for 30 sec. Insoluble particles were removed by centrifugation of the lysates at 5,200 x g at 4°C for 20 minutes using a small rotor (JA) of the Beckmann, Avanti^{TM} J-25. The supernatant was kept at -80°C after adding glycerol (10% v/v) for preservation. This supernatant contains soluble p53 protein (2%) important for future work. The pellets were resuspended twice in 5 ml of buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA, 10 mM NaCl, 1 mM PMSF, 0.5% v/v Triton-X100) to wash the protein, then centrifuged again at 5,200 x g at 4°C for 20 minutes

using a small rotor (JA) of Beckmann Avanti^{TM} J-25. The pellets were solubilised in 5 ml of 5 M guanidine hydrochloride, 50 mM Tris-HCl pH 8.0, 0.005% v/v Tween 80 by mixing on a rotating wheel at 4°C overnight in the cold room and then centrifuged at 5,200 x g at 4°C for 20 minutes using a small rotor (JA) to remove insoluble material. The supernatant was transferred into a fresh tube and diluted to a final concentration of 1M guanidine hydrochloride (40 ml of a mixture of 50 mM Tris- HCl pH 8.0, 0.005% v/v Tween 80, 2 mM reduced glutathione and 0.02 mM oxidized glutathione) (Sigma, Dorset, UK) and placed on a rotating wheel at 4°C overnight. Afterwards the protein was dialysed for at least 6 hours at 4°C using 2 litres of the following buffers

a) 50 mM HEPES pH 8.0, 300 mM NaCl, 0.005 % v/v Tween 80

b) 50 mM HEPES pH 8.0, 250 mM NaCl, 0.005 % v/v Tween 80

c) 50 mM HEPES pH 8.0, 200 mM NaCl, 0.005 % v/v Tween 80

d) 50 mM HEPES pH 8.0, 150 mM NaCl, 0.005 % v/v Tween 80

The resolubilised protein was centrifuged at 5,200g for 20 minutes at 4°C in the Beckmann, Avanti^{TM} J-25 using the JA rotor. A sample was transferred, 100 µl from the supernatant which is the resolubilised protein, and added the same volume of 2 x loading dye supplemented with 1/10 volume of 100 mM DDT, then testing the purity by SDS-PAGE gel analysis. For the remaining volume of the dialysed protein, 10% v/v glycerol was added for preservation purposes and stored at -80°C for further use.

3.7 Nitration of p53 protein by peroxynitrite

An aliquoted of 0.2 ml of p53 protein (0.2 mg/ml) was transferred into two glass test tubes, for nitration with 0.1 mM PN (174 mM) (Batch no # B62483, Cal Biochem, UK) and with 0.5 mM PN (174 mM). One additional test tube with each 0.2 ml of BSA protein (0.2 mg/ml) was included as a positive control (nitration with 0.5 mM PN). All of the three glass tubes were placed in a 37°C shaking water bath for 30 minutes. After this initial incubation the nitration process was started by adding 0.1 µl and 0.57 µl of PN to the appropriate glass tubes at final concentration of 0.1 mM and 0.5 mM of PN, respectively. While adding PN, a pinch stirrer was used to allow maximum reaction of PN with both p53 and BSA proteins. Then 10 x SDS loading dye was added to the p53 and BSA protein samples and the samples were transferred into a water bath (100°C) for 4 minutes. Immediately the samples were loaded into a 10% w/v SDS-PAGE gel and gel electrophoresis was started (80 volts for about 2 hours). Western blotting followed for detection of nitro tyrosine (Upstate, Lake Placid, U.K) at a concentration of 1:1000 for 1 hour at RT. Rabbit anti-mouse IgG HRP (Dacocytomation, Glostrup, Denmark) was used as a secondary antibody for 1 hour at RT (dilution 1:1000).

3.7.1 Dose response for nitration of p53 by peroxynitrite

To test the nitration in a concentration dependent manner, 0.1ml p53 protein was used, starting in six different glass test tubes. Then p53 protein in each glass tube was diluted with 50 mM HEPES, 50 mM NaCl and 0.005% v/v tween 80 to a final volume of 500 µl. These tubes were classified into, nitrated p53 with PN at final

concentrations 20 µM, 40 µM, 60 µM, 80, µM and 100 µM. Nitrated BSA (0.05 mM PN) served as a positive control. All samples were analysed by Western blotting.

3.7.2 Time response for nitration of p53 by peroxynitrite

The main purpose of this experiment was to find out, how long it takes for PN to act with proteins. In order to approach this question 0.1ml p53 was nitrated with 0.1mM PN over time (1 sec, 5 sec, 15 sec, 30 sec, 60 sec and 90 sec). Previously nitrated p53 (0.1mM PN) was used as a positive control. P53 protein was diluted with 50 mM HEPES, 50 mM NaCl and 0.005% v/v tween 80 to a final volume of 500 µl as performed in the previous section. This experiment was completed by Western blotting.

3.7. 3 Immunoprecipitation of the mutant p53 of HT-29 cells by using protein G agarose

To an aliquoted of 1 ml of HT-29 frozen cell suspension containing mutant p53 (kept in -80°C, suspended in 1 ml PBS) 50 µl of 1 x protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany), 10 µl of 10% v/v Igepal and 10 µl of 0.5 M EDTA were added. Incubation for 45 minutes on ice was followed with occasional mixing. Then the suspension was centrifuged for 15 minutes at 14,000 x g at 4°C. 20 µl of the supernatant was transferred to a fresh tube and 20 µl of 2 x loading dye and 4 µl DDT (100 mM) was added. Prior to loading the samples into a gel, they were boiled in a water bath for 4 minutes. 15 µl of these samples served as an internal control. Additionally p53 (resolubilized from *E. coli*, strain and BL21/DE3 pT7.7Hup53 that were kept in -80°C) was diluted by 1:30 using 1 x loading dye and 6 µl 100 mM DDT was added followed by heating for 4 minutes before loading into the gel.

Preclearing stage

A 30 µl of protein G agarose (Sigma, Dorset, UK) was added to the protease inhibitor, 10% v/v Igepal and 0.5 M EDTA and kept in ice for 20 minutes to ensure that same condition. After mixing the solution was centrifuged at 14,000 x g for 1 minute in a micro centrifuge. A 700 µl of supernatant was mixed with protein G agarose and kept on ice for 45 minutes. Then it was centrifuged at 14,000 x g for 3 minutes in a microcentrifuge and the pellets (protein G agarose) were transferred into fresh tubes followed by washing twice with 20 µl 1 x PBS, 20 µl HSB (high salt buffer, 0.5 M NaCl, 5 mM EDTA and 50 mM Tris-HCl, pH 8.0). After a final centrifugation step (14,000 x g for 3 minutes) 15 µl was loaded into the gel to probe whether protein G agarose bound to p53 protein. 15 µl of the supernatant was loaded into the gel to test for unbound p53 protein.

Clearing stage

After removing protein G agarose by centrifugation, 700 µl of supernatant was supplemented with 4 µl of antibody DO-1 and kept on ice for 1 hour under occasionally mixing. Thereafter, 40 µl of protein G agarose was added to the antibody labelled supernatant and incubated on a mixing rotor in a cold room (4°C) for 90 minutes. This step was followed by centrifugation at 14,000 x g for 3 minutes in a microcentrifuge. 50 µl of the supernatant was added to 50 µl 2 x loaded dye, 8 µl 100 mM DDT and boiled for 4 minutes in the water bath. Then 15 µl was loaded onto the gel in order to test whether there was still p53 protein in the supernatant. The pellets were washed twice with HSB, then centrifuged at 14,000 x g for 3 minutes and 15 µl of the resulting supernatant was loaded onto the SDS-PAGE gel. This was an important step to ensure whether p53 protein was still present after

washing. Also 15 µl of the pellets (p53 protein, DO-1 and protein G agarose) was loaded into the gel to determine whether DO-1 labelled protein G agarose contained p53 protein.

3.8 Detection of mutant type p53 from HT-29 cells

3.8.1 Identification of immunoprecipitated mutant p53 protein from HT-29 cells

Serial volumes from the sample were made by loading 2 µl, 4 µl, 6 µl, 15 µl and 20 µl onto the gel to demonstrate different concentrations of the immunoprecipitated mutant type p53 (HT-29 cell). The SDS-PAGE gel was run as described previously and prepared for Western blotting.

3.8.2 Immunoprecipitation of the nitrated mutant p53 protein of HT-29 cells using protein G agarose

HT-29 cells were cultured in three T75 culture flasks and after 6 days these four large flasks were trypsinized with 6 ml. After trypsinization, 4 ml RPM1, 1640 media (Invitrogen, Paisley, UK) were used to wash all cells off. The complete cell suspension was transferred into separate 1.5 ml Eppendorf tubes and centrifuged at 7,000 x g for 10 minutes. The supernatant was discarded and the pellets were washed twice with PBS, previously kept in a water bath at 37°C. Thereafter, the pellets obtained from each T75 culture flasks were resuspended in 1 ml of PBS. Finally there were 4 ml of cell lysates, nitrated cell lysates with 0.5 mM PN and cell lysates treated with decomposed 0.5 mM. 1ml of cell lysate was not nitrated to be used as a negative control. All cell lysates (HT-29 cells) were subjected to immunoprecipitation using protein G agarose as described in detail. SDS-PAGE gel and Western blotting were prepared as described previously. Stripping procedures were carried out to incubate the membrane in the washing solution (5% w/v milk, 0.4% tween 20, 1x PBS) supplemented with 2% v/v SDS and 5% v/v 2 β-mercaptoethanol for 45 minutes at 80°C followed by a wash for 30 minutes and

exposed to the primary anti-nitro tyrosine antibody (1:1000) for 1 hour at RT. Thereafter, the membrane was washed for 30 minutes and incubated with the secondary rabbit anti mouse IgG HRP antibody (1:1000) for 1 hour at RT followed by a final wash with the same washing solution for 30 minutes and the result was visualised with ECL solution.

3.8.3 Dose response for nitrated mutant p53 protein

Separate aliquots (5 ml each) were obtained from HT-29 cells as described above. The nitration process for HT-29 cells was performed by treating 4 ml of the cell lysate with 10 μM PN, 50 μM PN, 100 μM PN and 250 μM PN. To test the effect of decomposed PN on the cell lysate, 1ml of the lysate was treated with decomposed 0.5 mM PN as described earlier. All lysates (HT-29 cells) underwent the immunoprecipitation process using protein G agarose as described in detail, followed by Western blotting and stripping procedures as described earlier.

3.9 Electromobility Shift Assay (EMSA)

3.9.1 Designing, radiollabeling and purification of the labelled oligonucleotides duplex

Oligonucleotides containing the 20-mer consensus DNA binding site of p53 (the forward) and their complementary sequences (the reverse) were ordered from Sigma Genosys.

5' -AGACATGCCTAGACATGCCT-3' (Forward)

5' –AGGCATGTCTAGGCATGTCT-3' (Reverse)

Both forward and reverse oligonucleotides were diluted to a concentration of 1 μg/μl with nuclease free water (Promega Corporation, Southampton, UK). The diluted oligonucleotides were mixed together in a 1:1 ratio and left at RT for 5 minutes, in order to form the oligonucleotide duplex. The end labelled process was carried out by adding 10 pmol of 5'-ends of oligonucleotides and 4 μl of [^{32}P] γ-ATP (stock: 9.25 MBq/50 μl, Perkin Elmer, UK) into the tube that contained ready-to-go T4 polynucleotide kinase (Amersham Pharmacia, Biotechnology, UK) and distilled water giving a final volume of 50 μl. Thereafter, the reaction mixture was incubated in a water bath for 38 minutes at 37°C and the reaction was stopped by adding 5 ml of 250 mM EDTA.

Radiolabelled oligonucleotides as well as unincorporated [^{32}P]γATP were separated by using spin columns (MicroSpin^{TM} G-25 columns, Active Motif, Belgium) by following the manufacture's protocol. To confirm the purity of the end-labelled oligonucleotide duplex, 4% non-denaturing polyacrylamide gel (32.22 ml H_2O, 5.33 ml of 30% acrylamide, 40 μl TEMED, 400 μl of 10% APS, 2 ml of 10 x TBE) (Sigma Diagnostics Inc., St. Louis, USA) was prepared and placed into a protein IIXI

vertical electrophoresis cell (Bioradcorporation, Hertfordshire, UK). The reaction mixture (2 µl of radiolabelled oligonucleotides, 4 µl of distilled water and 4 µl of 6 x blue / orange loading dye (Promega corporation, Southampton, UK) was loaded and the autoclaved 10 x TBE buffer (pH8.3) was used as running buffer (108 g Tris base, 55 g boric acid, 9.3 g disodium EDTA and distilled water to a final volume of 1000 ml).

The gel was electrophoresed at 150 V until the dye front reached a third of the way from the top. Gels were dried by a dryer (Drygel Sr. Slab gel dryer, Hoeffer Scientific Instruments, SanFrancisco, USA) for about 1.5 hour at 80˚C. Finally the band for endlabelled oligonucleotide duplex was detected by exposing the dry gel to Kodak X-Omat film for 24 hours followed by standard ECL technique.

3.9.2 Effect of H_2O_2 and peroxynitrite (PN) on p53-DNA binding capacity

The effect of H_2O_2 and PN on p53-DNA binding was investigated separately in two experiments. The combined effect of PN / H_2O_2 on p53-DNA-binding was shown in one single experiment. 250 ng/µl of active wild type p53 protein (expressed in insect cells using baculovirus expression system, 500 ng/µl, Active Motif, Belgium) was treated with different H_2O_2-concentration (100 µM, 200 µM, 300 µM, 500 µM, 800 µM, 1 mM) for 2 minutes and then the excess H_2O_2 was removed by adding 5 ml of 5 mg/ml catalase (Sigma, Dorset, Poole, UK). The treated p53 was transferred into a new Eppendorf tube containing 4 µl of the purified radiolabelled oligonucleotide duplex and p53-DNA binding buffer (Hupp, Meek et al. 1992) to complete the reaction volume to 20 µl. Native active p53 protein and 4 µl of the purified radiolabelled oligonucleotide were used as a positive control. Resolubilised p53 (expressed in *E. Coli*) and 4 µl of the purified radiolabelled oligonucleotide was used

as a negative control. All samples were incubated at 0°C on ice for 36 minutes. The reaction products were loaded onto pre-cooled 3% polyacrylamide native gel (33.56 ml H_2O, 4 ml of 30% acrylamide, 40 µl TEMED, 400 µl of 10% APS, 2 ml of 10 x TBE) (Sigma Diagnostics Inc., St. Louis, USA) in 0.5XTBE running buffer followed by electrophoresis at 196 V until the dye marker reached half way down the gel. Thereafter the gel was dried and the bands were visualised on a Kodak X-Omat film. In order to investigate the effect of PN on p53, peroxynitrite (174 mM Calbiochem, Darmstadt, Germany) diluted in 0.1 M NaOH (Oberprieler 2007) was used as described for H_2O_2. To study the effect of the combination of PN/H_2O_2 on DNA-binding, p53 was first treated with H_2O_2 as described above followed by treatment with PN.

3.10 Molecular structural computer modelling of the native and oxidized/nitrated p53-DNA binding domain

In order to provide a better insight into the effect of H_2O_2, PN and the combination of both on p53-DNA binding the molecular modelling program "HyperChem" (Hypercube, Inc, Gainesville, FL, USA), Deep View analysis was used to compare and investigate the changes that could happen after oxidation and nitration to p53-DNA binding capacity. The crystal structure of human p53 was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/hoe/home.do.) accession number 1TUP. This work was carried out in collaboration with Dr N. Gibbons, University of Bradford.

4.0 RESULTS

4.1 The presence of p53 in the human epidermis

4.1.1 Up-regulation of p53 protein in association with low catalase expression in patients with vitiligo

It was previously demonstrated that p53 expression is up-regulated in vitiligo (Schallreuter, Behrens-Williams et al. 2003). Moreover, this up-regulated p53 is a wild type and functioning protein (Schallreuter, Behrens-Williams et al. 2003). The current study was planned to test whether p53 protein expression in vitiligo was correlating with H_2O_2-mediated oxidative stress levels. Since catalase is considered as a biomarker for oxidative stress (Schallreuter, Bahadoran et al. 2008), these levels formed the base as internal standard for assessment of H_2O_2 stress in all patients (n=10) and controls (n=4). In order to minmize variabelity, skin samples from the same patients were used throughout all investigations.

The *in situ* results show high p53 protein expression throughout the epidermis of vitiligo lesional (f) and vitiligo non lesional skin (j) compared to healthy controls (b). In addition we show that p53 is over expressed in the nucleus of vitiligo lesional (h) and vitiligo non lesional skin (l) compared to controls (d) **(Figure 16A)**.

Image analysis of p53 protein expression in the whole epidermis revealed that protein expression is significantly increased in both vitiligo lesional (*** $p<0.001$, mean ± SD) and vitiligo non lesional (** $p<0.01$, mean ± SD) epidermis compared to healthy controls **(Figure 16B/C)**. There is no significant difference between lesional

and non lesional skin ($p>0.05$). This observation confirmed earlier results (Schallreuter et al 2003, van den Wijngaard et al 2000).

In order to correlate the expression of p53 levels to individual H_2O_2-mediated oxidative stress in trhe same samples, epidermal catalase levels were evaluated as well. Catalase is significantly lower in vitiligo lesional and non lesional epidermis (*** $p<0.001$, mean ± SD), when compared to healthy controls. This result is in agreement with H_2O_2-mediated stress in vitiligo as documented earlier (Schallreuter et al 2008).

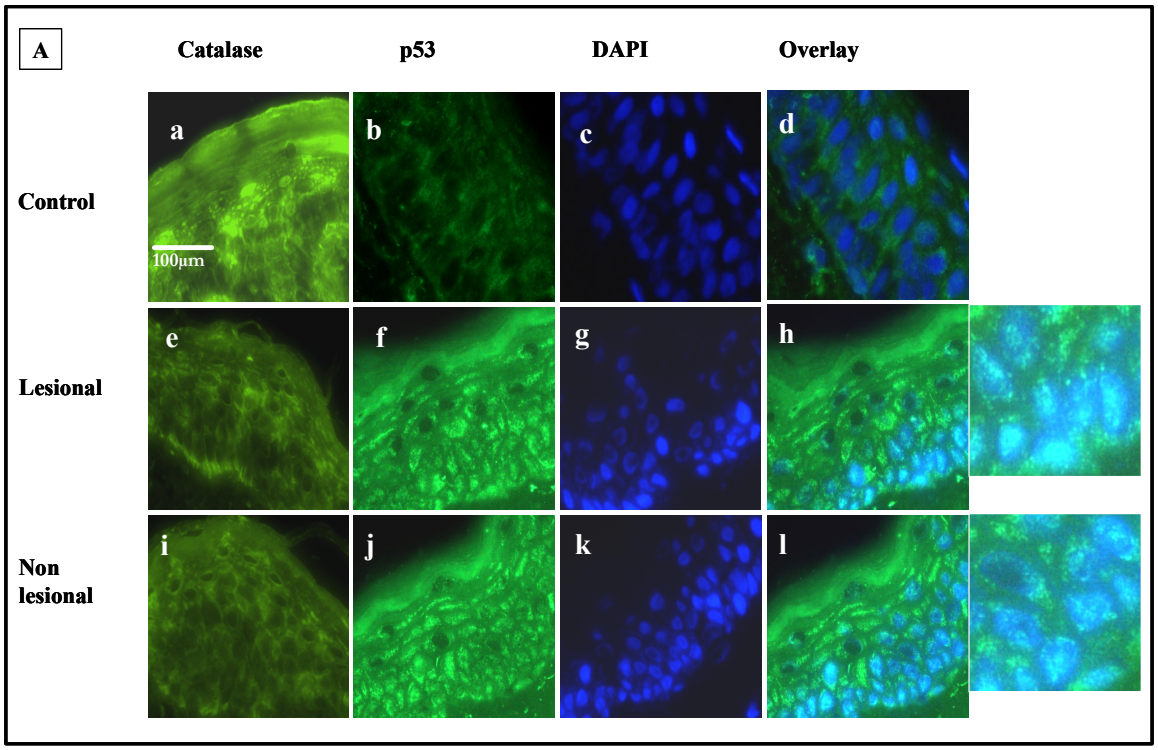
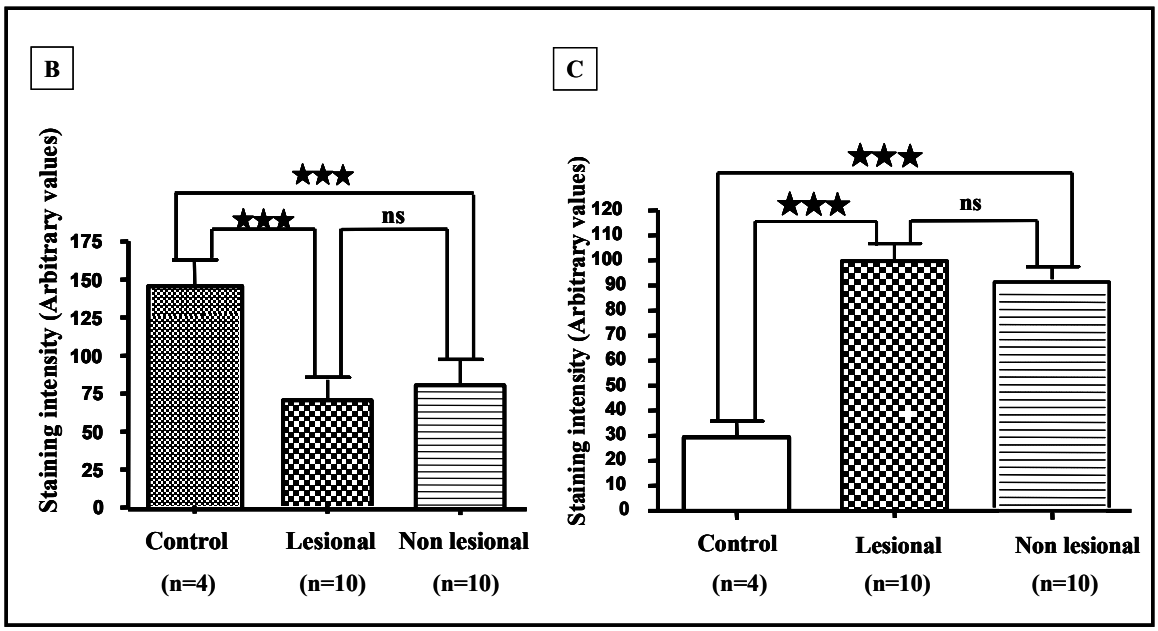


Figure 16
Increased epidermal p53 expression in patients with vitiligo in association with low catalase levels
(A) Immuno fluorescence staining (FITC labelling) shows over expression of p53 protein in vitiligo lesional (n=10) (f) and non lesional skin (n=10) (j) compared to healthy controls (n=4) (b); skin phototype III (Fitzpatrick classification). Low levels of catalase in vitiligo lesional (e) and non lesional skin (i) compared to healthy controls (a). The nucleus of epidermal cells of vitiligo lesional (h) and non lesional (l) exhibits a high expression level of p53 protein compared to controls (d). c, g and k presents DAPI for healthy control, vitiligo lesional and non lesional respectively. Magnification x 400, scale bar 100 µm.



(B) Image analysis of catalase. Values are the mean ± SD (*** p<0.001).
(C) Image analysis of p53 protein. Values are the mean ± SD (*** p<0.001).

4.1.2 Confirmation of over expression of p53 in vitiligo by Western blot

In order to further support the *in situ* findings on up-regulation of p53 in patients with vitiligo compared to healthy controls, Western blotting was carried out in epidermal suction blister tissue from 4 patients **(Figure 17A)**. The results support the *in situ* results of p53 protein over expression in these cell extracts (lanes 3-9) compared to normal healthy control (lane 2).

Moreover, epidermal p53 is up-regulated in lesional and non-lesional skin of patients with vitiligo in the presence of epidermal H_2O_2 levels in the mM range as evidenced by the corresponding low catalase levels of the patients examined.

One important take home message emerges from this study. Up-regulated p53 is not only present in the lesional epidermis. This result corresponds to almost all results which have been collected over many years (Schallreuter et al 2008) pointing to a general mechanism involved in this disease.

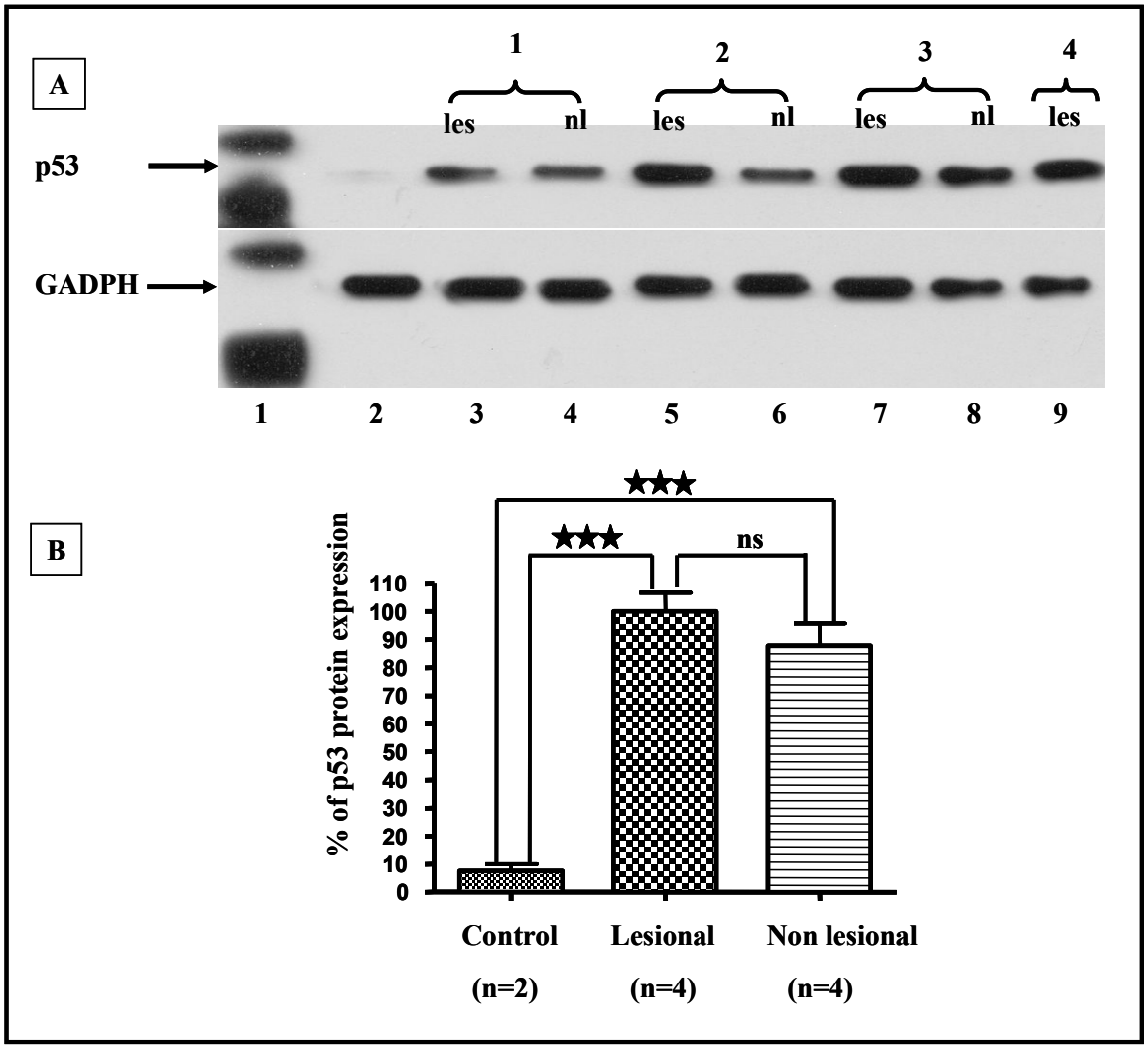


Figure 17
Epidermal suction blister tissues exhibit up-regulated p53 protein in vitiligo
(A) Western blot showing an increase in p53 protein expression in patients with vitiligo (lanes 3-9) compared to healthy controls (lane 2). The numbers on top of Western blot identify patient's numbers (4). Lane 1 is a protein ladder; GADPH was used as a loading control.
(B) Densitometry analysis of the bands reveals significantly higher levels of p53 protein after re-probing the blots with GADPH antibody (blots are ±SD). (*** $p<0.001$).

4.1.3 H_2O_2 does not affect the epitope of the p53 antibody

In order to exclude a possible effect of H_2O_2 on p53, we performed dot blot analysis using purified p53 (0.2 µg) from *E. Coli* in the presence of H_2O_2-oxidised protein. P53 was exposed to various H_2O_2 concentrations ranging from 1 to 100 x10^{-3}M for 1 hour at RT prior to analysis. The results **(Figure 18)** show that the epitope binding site for the antibody is not affected by H_2O_2.

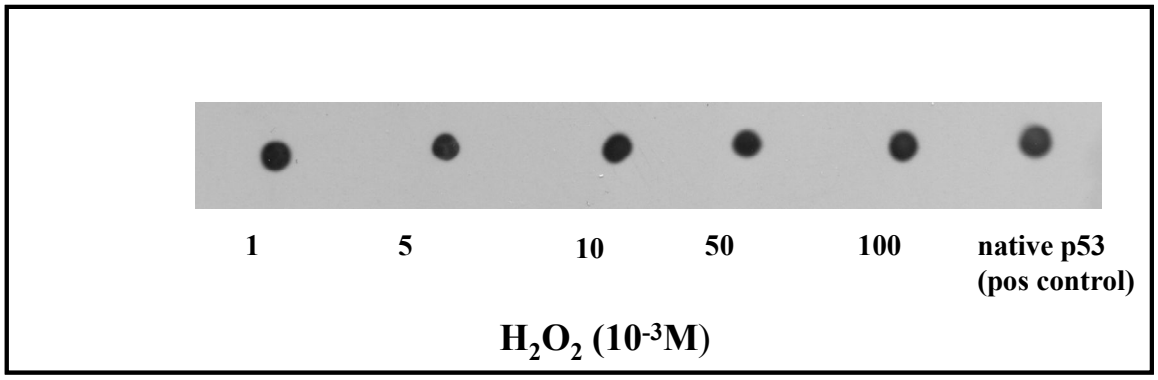


Figure 18
P53 protein epitope is not affected by H_2O_2
Dot blot proves that H_2O_2 does not affect immune-reactivity of the p53 protein epitope of the primary antibody used (DO-1, 1:15000)

4.1.4 Increased *in vitro* expression of p53 protein in vitiliginous melanocytes

Since melanocytes are the target cells in vitiligo we wanted to know whether p53 protein levels are also increased under *in vitro* conditions. Our immuno-reactivity results revealed that p53 protein is highly expressed in the nucleus of vitiliginous melanocytes **(Figure 19f)** whereas p53 is only expressed in the cytoplasm in healthy control cells **(Figure 19c)**. This result indicates that p53 is even up-regulated under *in vitro* conditions.

Together with the *in situ* and the Western blot data, it is tempting to invoke a very central role for p53 as master conductor in vitiligo.

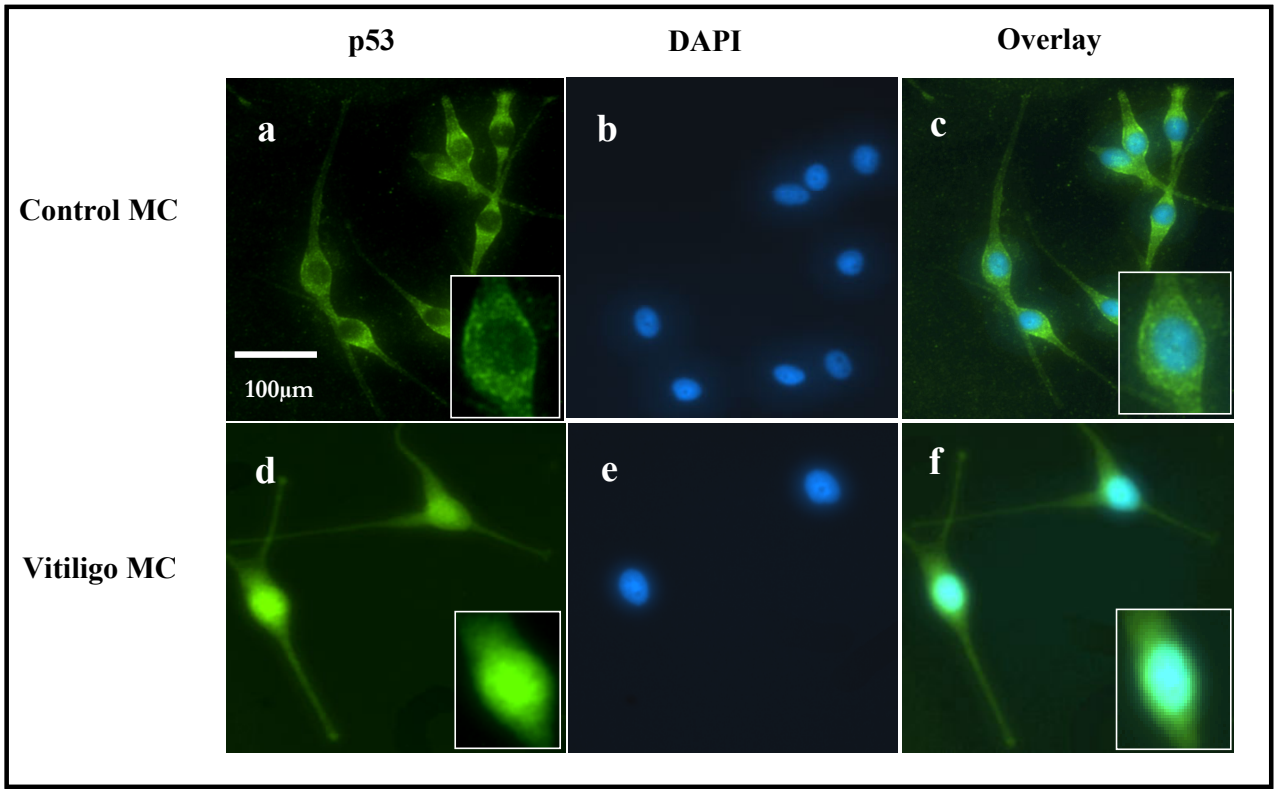


Figure 19
Strong cytosolic and nuclear p53 expression in vitiligo melanocytes
In vitiligo melanocytes (d) p53 protein is present throughout the cytosol and the nucleus compared to healthy control melanocytes where p53 expression is weaker and is more distributed in a granular pattern (a). (b) and (e) represent DAPI for control and vitiligo melanocyte respectively. Overlay with DAPI indicates strong nuclear expression in vitiligo (f).Magnification x 400, scale bar 100 µm.

4.2 Detection of two mdm2 isoforms in vitiligo

4.2.1 Normal expression of $p90^{MDM2}$ in patients

Given that p53 is up-regulated in vitiligo, we next looked into the degradation pathway of this protein. $P90^{MDM2}$ plays a crucial role in regulation of p53 in the cell because it is important for the nuclear signal transduction pathway. Therefore, we employed again *in situ* and *in vitro* immuno-reactivity staining in the corresponding skin samples. The *in situ* results show epidermal $p90^{MDM2}$ expression in normal healthy controls (a), vitiligo lesional (d) and non lesional (g) skin. Moreover, we found that $p90^{MDM2}$ expression is more pronounced in the nucleus **(Figure 20A)**. Image analysis shows no statistically significant difference between patients and controls ($p>0.05$, mean ± SD) (**Figure 20B).** This result is not influenced by the presence of high epidermal H_2O_2 levels as analysed in comparison to the corresponding catalase levels in the same samples (data not shown). Based on these results we can conclude that $P90^{MDM2}$ expression is not affected by H_2O_2 in vitiligo.

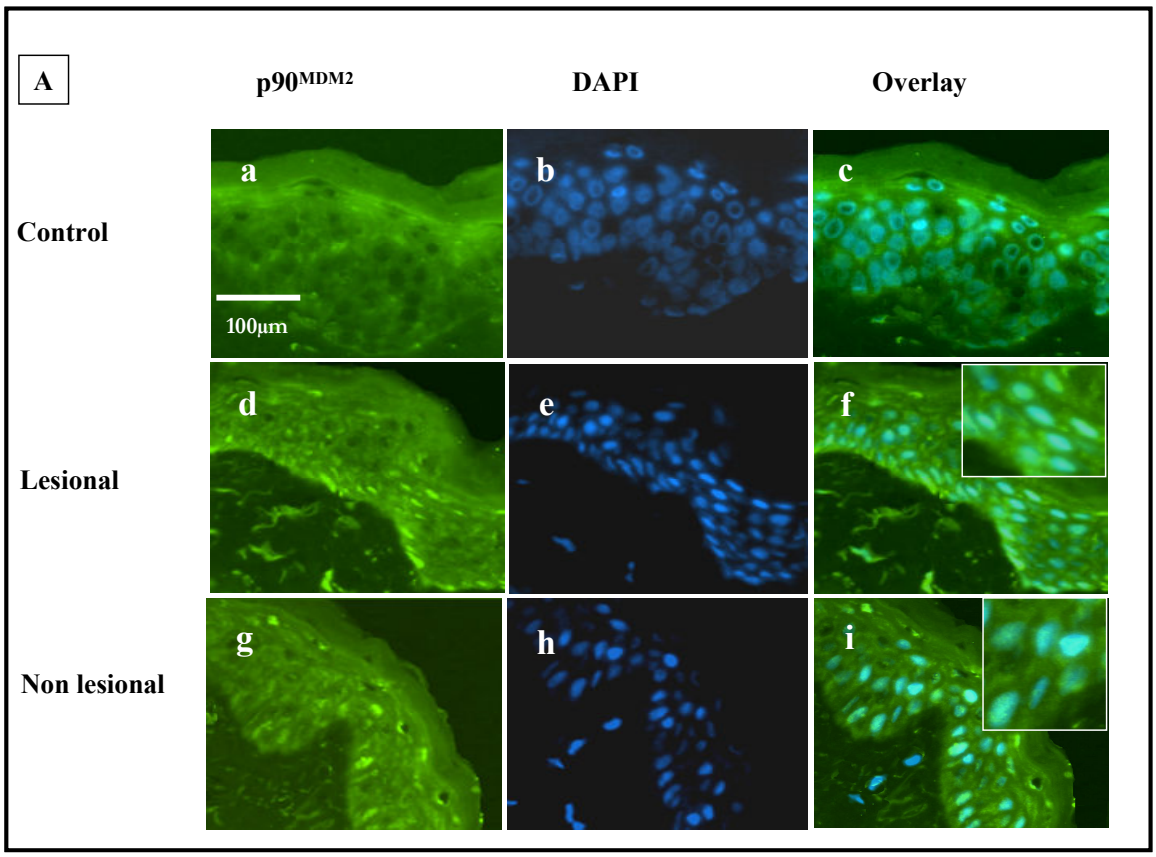
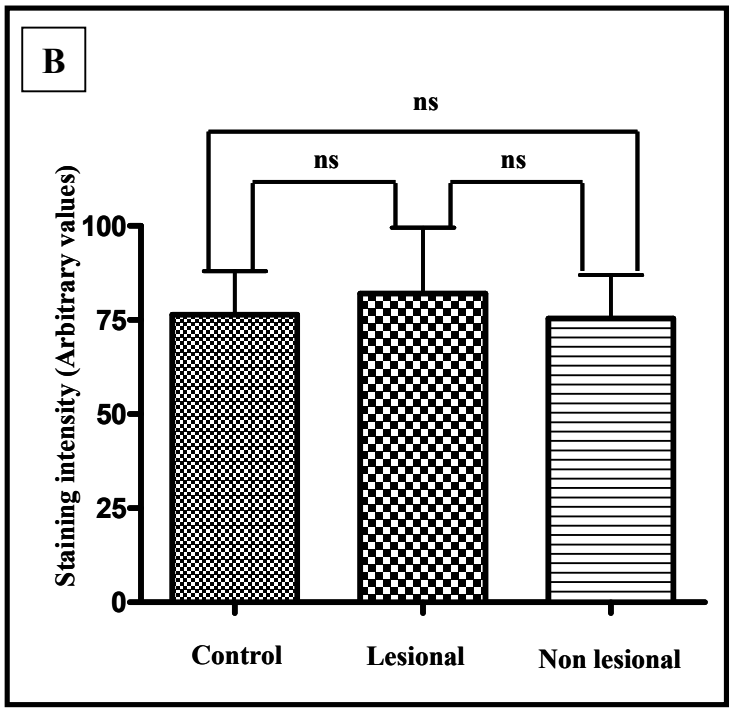


Figure 20
Expression of p90 ^{MDM2} is not altered in patients with vitiligo compared to healthy controls
(A) Immuno-reactivity staining shows no significant difference in p90^{MDM2} expression in vitiligo lesional (n =10) (d), vitiligo non lesional (n=10) (g) compared to healthy controls (n=4) (a); skin phototype III (Fitzpatrick classification). The expression of p90^{MDM2} is also found in the nucleus as shown by overlay with DAPI. Magnification x 400, scale bar 100 µm.



(B) Image analysis Average fluorescence intensity reveals that there is no significant increase in $p90^{MDM2}$ expression ($p>0.05$).

4.2.2 *In vitro* expression of $p90^{MDM2}$ in vitiliginous melanocytes compared to controls

In order to follow $p90^{MDM2}$ expression under *in vitro* conditions, immuno fluorescence staining for both vitiligo and healthy control melanocytes was processed. Again, $p90^{MDM2}$ expression is the same in vitiligo melanocytes and normal melanocytes **(Figure 21a and d** respectively**)**. Moreover, the protein is strongly expressed in the nucleus **(Figure 21c and f** respectively**)**. The current observations would favour that $p90^{MDM2}$ promotes degradation of p53 in the nucleus through an E3 ubiquitin ligase mechanism.

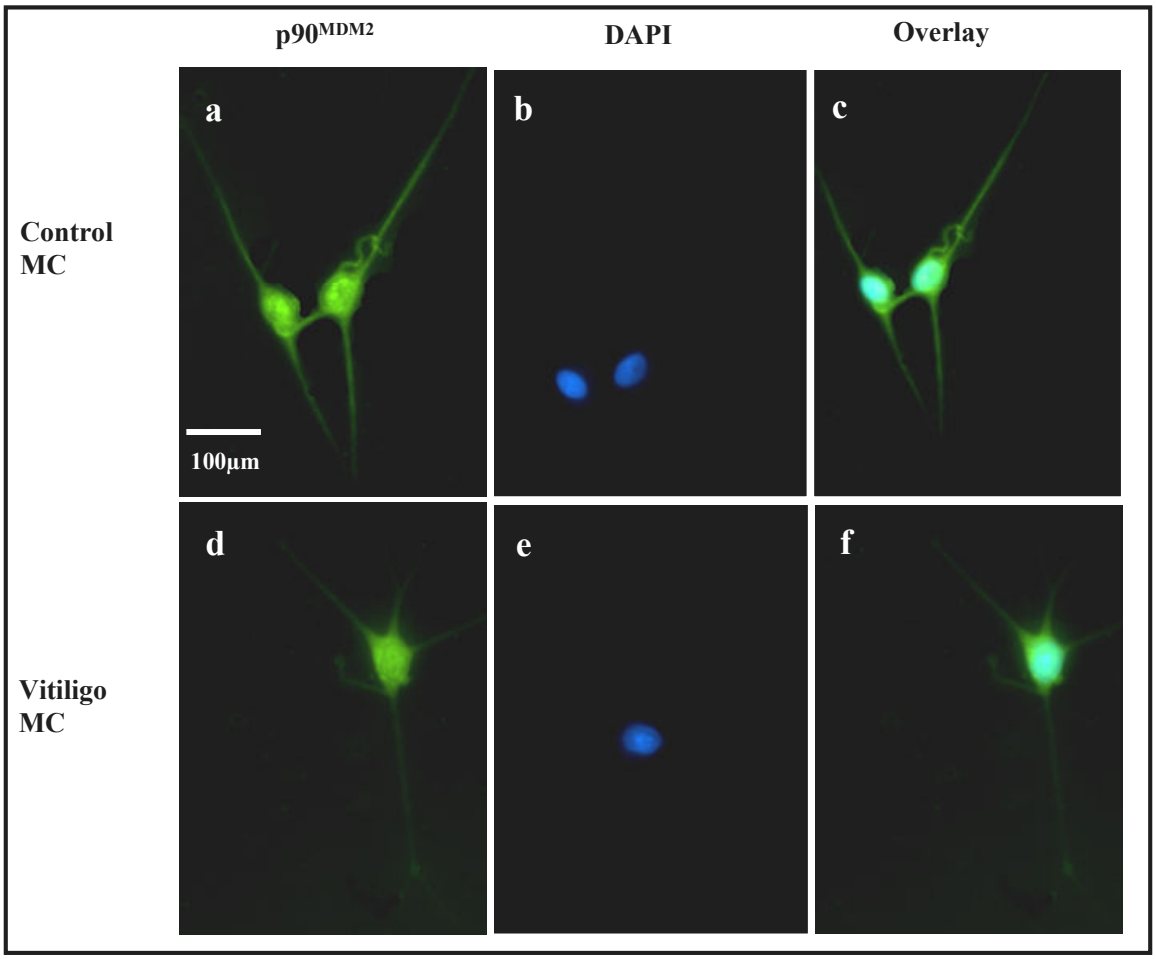


Figure 21
$p90^{MDM2}$ immuno-reativity shows the same expression level in vitiligo and control melanocytes
Normal melanocyte (a) and vitiligo melanocyte (d). Both normal melanocyte (c) and vitiligo melanocyte (f) induce this protein in their nucleus as shown by the overlay with DAPI. Magnification x 400, scale bar 100 µm.

4.2.3 Increased $p76^{MDM2}$ level in patients with vitiligo compared to healthy controls

The mdm2 protein is a nuclear protein that regulates p53 function with its capacity to suppress the transcriptional activity of the p53 protein. We confirmed that p53 is not degraded but it is over expressed and functional in patients with vitiligo (Schallreuter et al 2003). Therefore, the next aim was to seek an explanation for why mdm2 protein does not degrade p53. It has been shown that $p76^{MDM2}$ has the ability to antagonize $p90^{MDM2}$ action i.e. degradation of p53. Hence, it was tempting to investigate the pattern of $p76^{MDM2}$ expression in a large series of patients with vitiligo by using *in situ* immuno fluorescence technique with a specific antibody for $p76^{MDM2}$ (SMP14, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, U.S.A). This staining was carried out again in comparison with the corresponding catalase levels in the same samples to assess the levels of oxidative stress (data not shown).

We found the presence of up-regulated epidermal $p76^{MDM2}$ in patients with vitiligo compared to healthy controls **(Figure 22A/B)**. This isoform was also found in the nucleus.

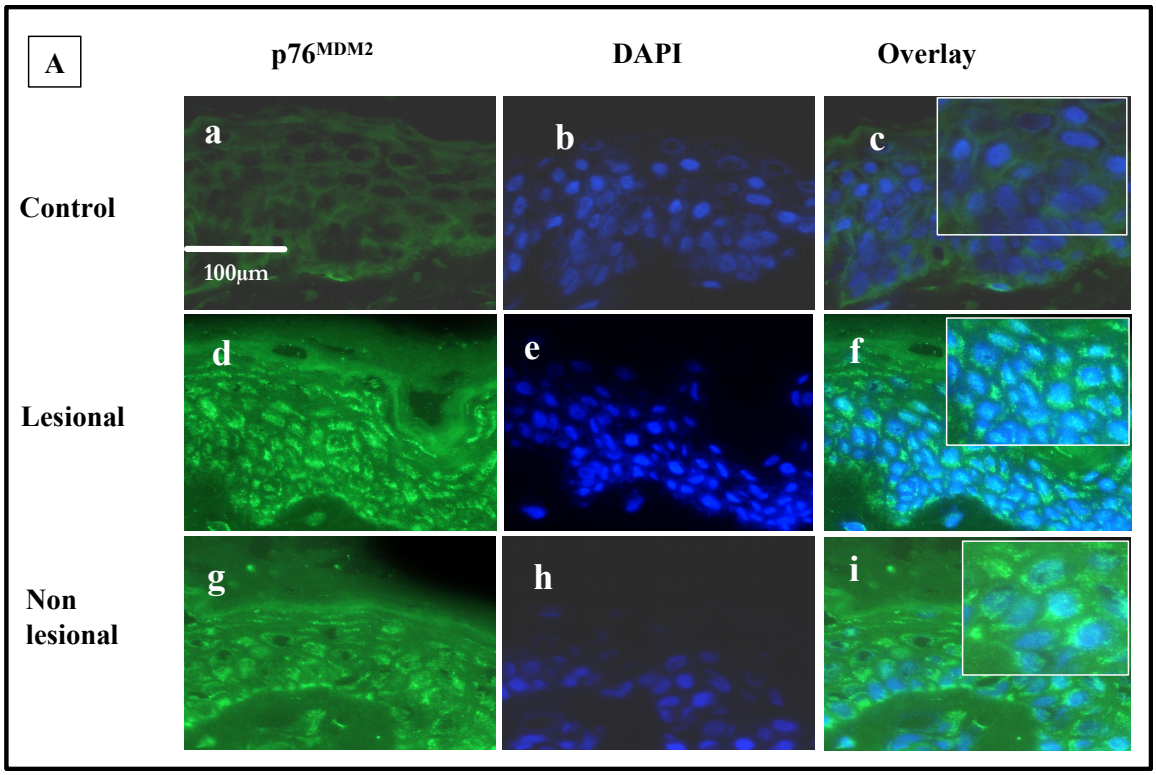
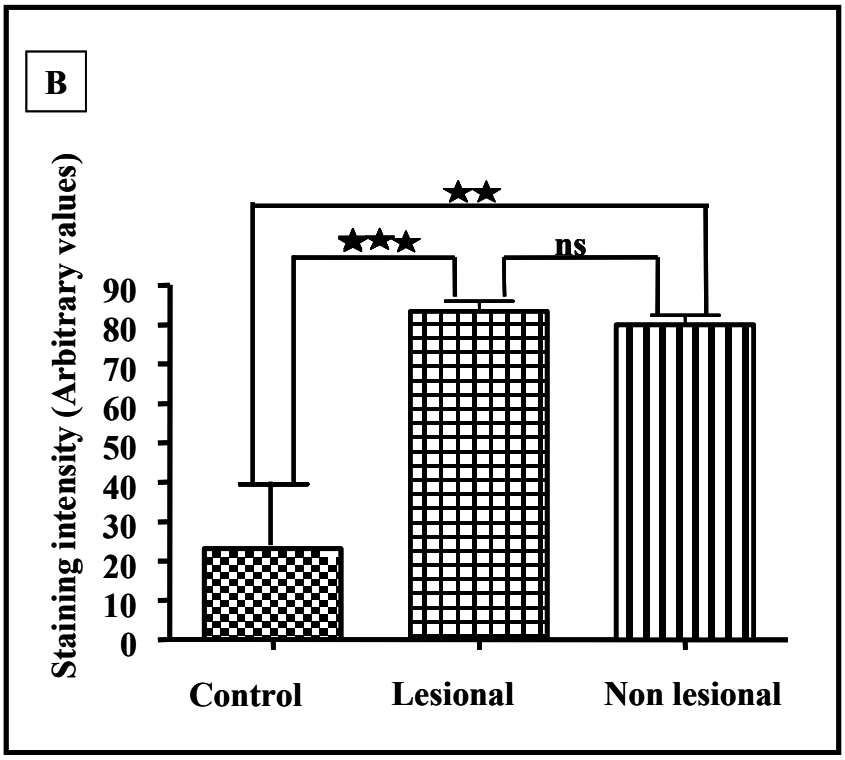


Figure 22
Patients with vitiligo reveal induced epidermal p76^{MDM2} levels
(A) *In situ* immuno fluorescence of p76^{MDM2} (FITC-labelled) reveals high protein expression of p76^{MDM2} in vitiligo lesional skin (d), non lesional skin (g) compared to control skin (a). (f) and (i) are overlays of vitiligo lesional and non lesional respectively with DAPI showing the presence of p76^{MDM2} in the nucleus which is absent in healthy controls (c). Magnification x 400, scale bar 100µm,



(B) Image analysis of p76^{MDM2} in vitiligo N.B. significant increase in the expression of p76^{MDM2} throughout vitiligo (n=10) skin compared to healthy controls (n=4) (*** $p<0.001$, mean ± SD, ** $p<0.01$, mean ± SD, ns = not significant). N.B. There is no difference between lesional and non lesional skin.

4.2.4 Epidermal cell extracts from patients with vitiligo reveal significantly higher $p76^{MDM2}$ protein levels

In order to further validate the immuno fluorescence data, Western blot analysis was performed in epidermal suction blister cell extracts from 3 patients with vitiligo and 3 healthy controls using the same $p76^{MDM2}$ antibody. $p76^{MDM2}$ was significantly stronger expressed in extracts from patients with vitiligo compared to healthy controls **(Figure 23)**. These results suggest that $p76^{MDM2}$ antagonizes $p90^{MDM2}$ leading in turn to stability and consequently to up-regulation of p53. Importantly, the up-regulation is present in lesional and non- lesional tissues.

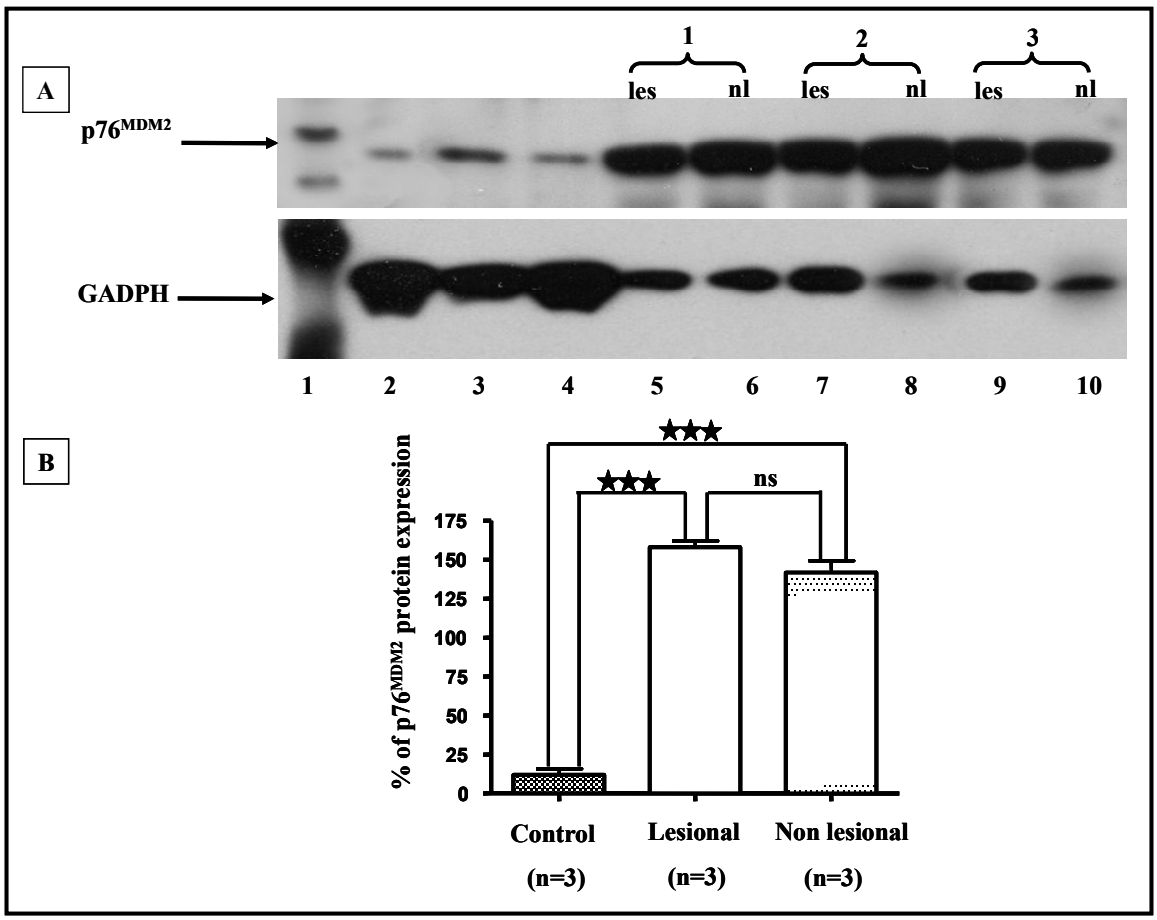


Figure 23
Over expressed p76^{MDM2} protein in vitiligo
(A) Western blot with SMP14 antibody Over expression of p76^{MDM2} in 3 patients with vitiligo compared to healthy controls. Lane 1 protein ladder, lanes 2-4 healthy controls and lanes 5-10 patients with vitiligo. GADPH was used as a loading control.

(B) Image analysis Evaluation of the bands was performed in relation to loaded protein (GADPH). The result demonstrates that p76^{MDM2} is up-regulated in vitiligo compared to controls (*** $p<0.001$).

4.3 Immuno-reactivity expression of iNOS and nitrated tyrosine in the human epidermis

4.3.1 Increased epidermal iNOS expression in patients with vitiligo as an indicator for the presence of NO

On one hand, it has been shown that NO can stabilize p53 (Forrester et al 1996, Messmer et al 1994 &1996). On the other hand it was shown that nitration affects p53-DNA binding (Calmes et al 1997, Cobbs et al 2001). Since patients with vitiligo have high levels of H_2O_2 in their epidermal compartments, it was tempting to explore the possibility of p53 oxidation/nitration. Therefore, we investigated the level of iNOS in the epidermis of patients and controls. In order to demonstrate the expression level of this enzyme, immuno fluorescence and Western blotting studies were used. Our *in situ* immuno fluorescence results show stronger expression levels throughout all patients with vitiligo lesional (d) and vitiligo non lesional skin (g) **(Figure 24A)** in comparison to healthy controls (a). Catalase was again used as internal control to monitor oxidative stress (data not shown). Statistical analysis for all patients used in this study, revealed significant higher levels of iNOS in both vitiligo lesional (** $p<0.01$, mean ± SD) and non lesional skin (* $p<0.05$, mean ± SD) compared to controls **(Figure 24B)**. There is no significant difference between vitiligo lesional and vitiligo non lesional skin in the expression level of iNOS ($p>0.05$, mean ± SD).

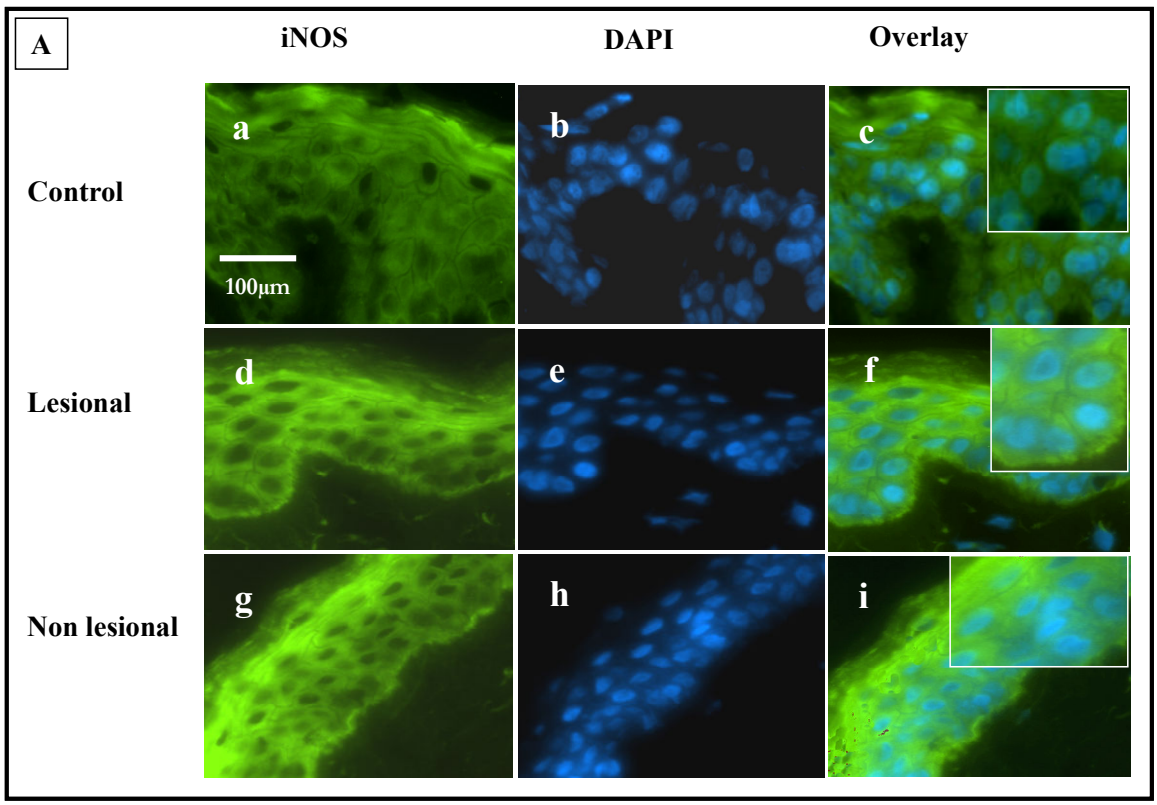
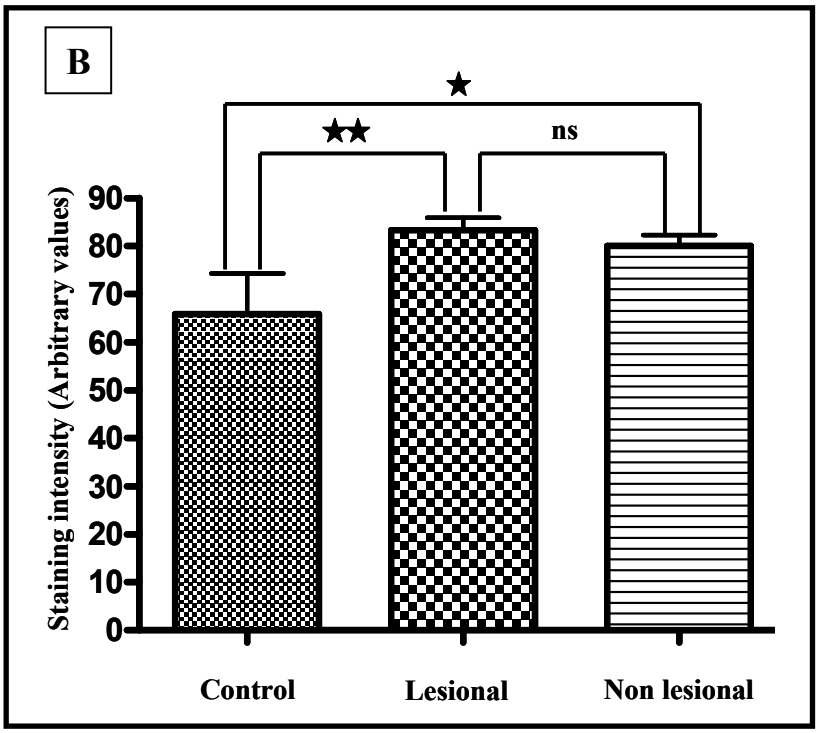


Figure 24
Increased *in situ* iNOS expression in patients with vitiligo
(A) *In situ* immuno fluorescence staining of iNOS (FITC-labelled) demonstrates high expression level of iNOS in vitiligo lesional (d), non lesional (g) compared to control (a). (f) and (i) overlay of vitiligo lesional and non lesional respectively with DAPI shows localization of iNOS in the nucleus compared to healthy control (c). (b), (e) and (h) represent DAPI for controls, lesional and non lesional skin respectively. Magnification x 400, scale bar 100µm.



(B) Image analysis showing a significant increase in the expression of iNOS in vitiligo lesional (** $p<0.01$) and non lesional skin (* $p<0.05$) compared to healthy controls.

4.3.2 More evidence for up-regulation of iNOS in vitiligo

The increased expression of iNOS in the epidermis of patients with vitiligo was confirmed by Western blotting in cell extracts from 5 patients with vitiligo and 2 controls. The results show that iNOS exhibits a high expression level in vitiligo (lanes 4-13) when compared to control (Lanes 2 and 3) **(Figure 25)**.

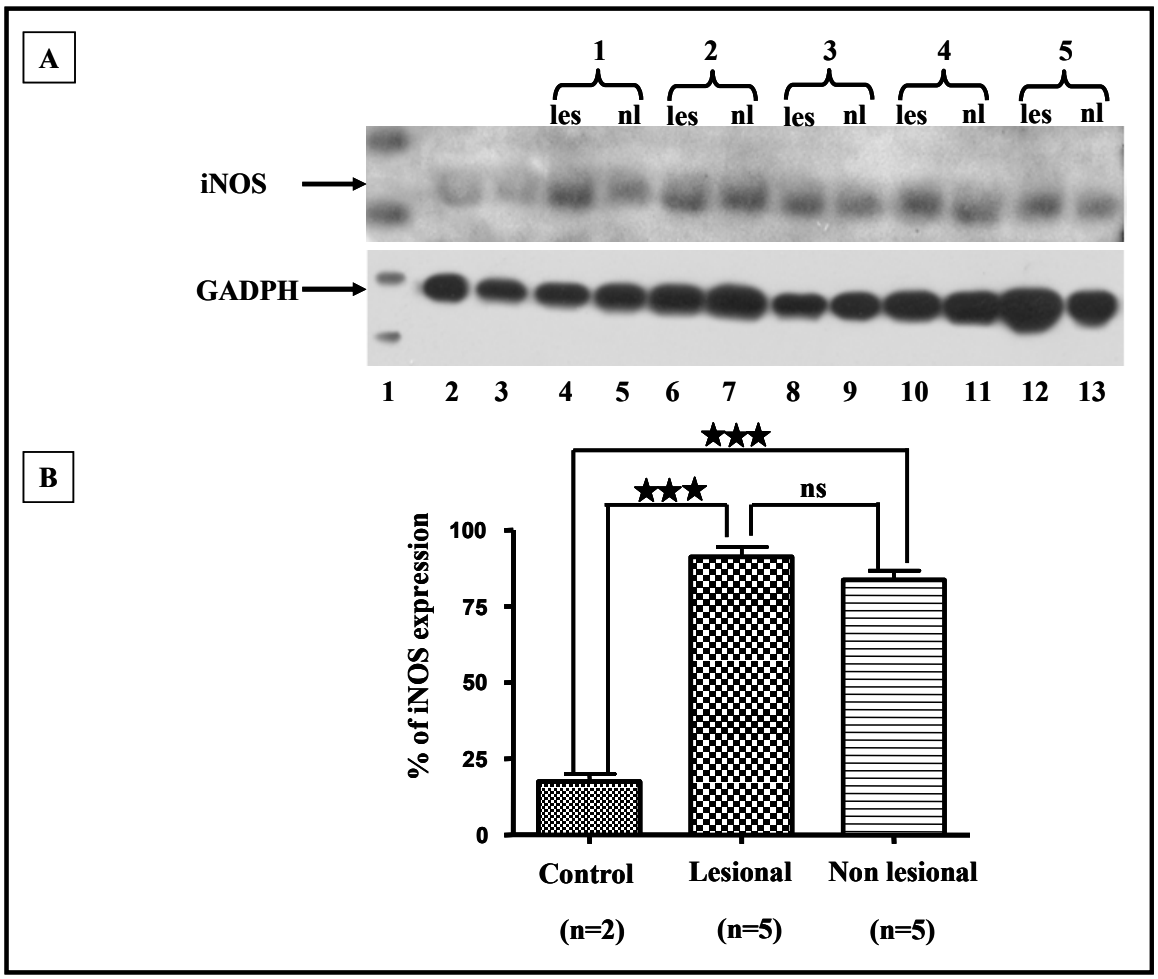


Figure 25
Induction of epidermal iNOS in patients with vitiligo
(A) As seen on the Western blot, iNOS levels are significantly different between 5 patients with vitiligo (lanes 4-13) and 2 controls (lanes 2 and 3). Lane 1 represents protein ladder. GADPH served as loading control.
(B) Densitometric measurements were performed to iNOS levels in correlation with loaded protein (GADPH). iNOS is significantly over expressed in vitiligo lesional compared to control (plots are mean ± SD). (*** $p<0.001$).

4.3 3 Vitiliginous melanocytes express iNOS under *in vitro* conditions

Our *in situ* previous data showed up-regulation of iNOS in the skin of patients with vitiligo. Moreover, this result was confirmed by Western blot. To explore whether this was also the case under *in vitro* conditions in vitiliginous melanocytes, we used again immuno fluorescence labelling. The result in **Figure 26** displays expression of iNOS, which is much more pronounced in the nucleus of melanocytes from patients **(Figure 26d)**, compared to control cells **(Figure 26a)**. Importantly, expression of iNOS is also present in the nucleus of vitiliginous melanocytes **(Figure 26f)** and control **(Figure 26c)**. Taken together, increased iNOS expression in vitiligo suggests increased NO production which in turn reacts with $O_2^{\cdot-}$ to produce $ONOO^-$.

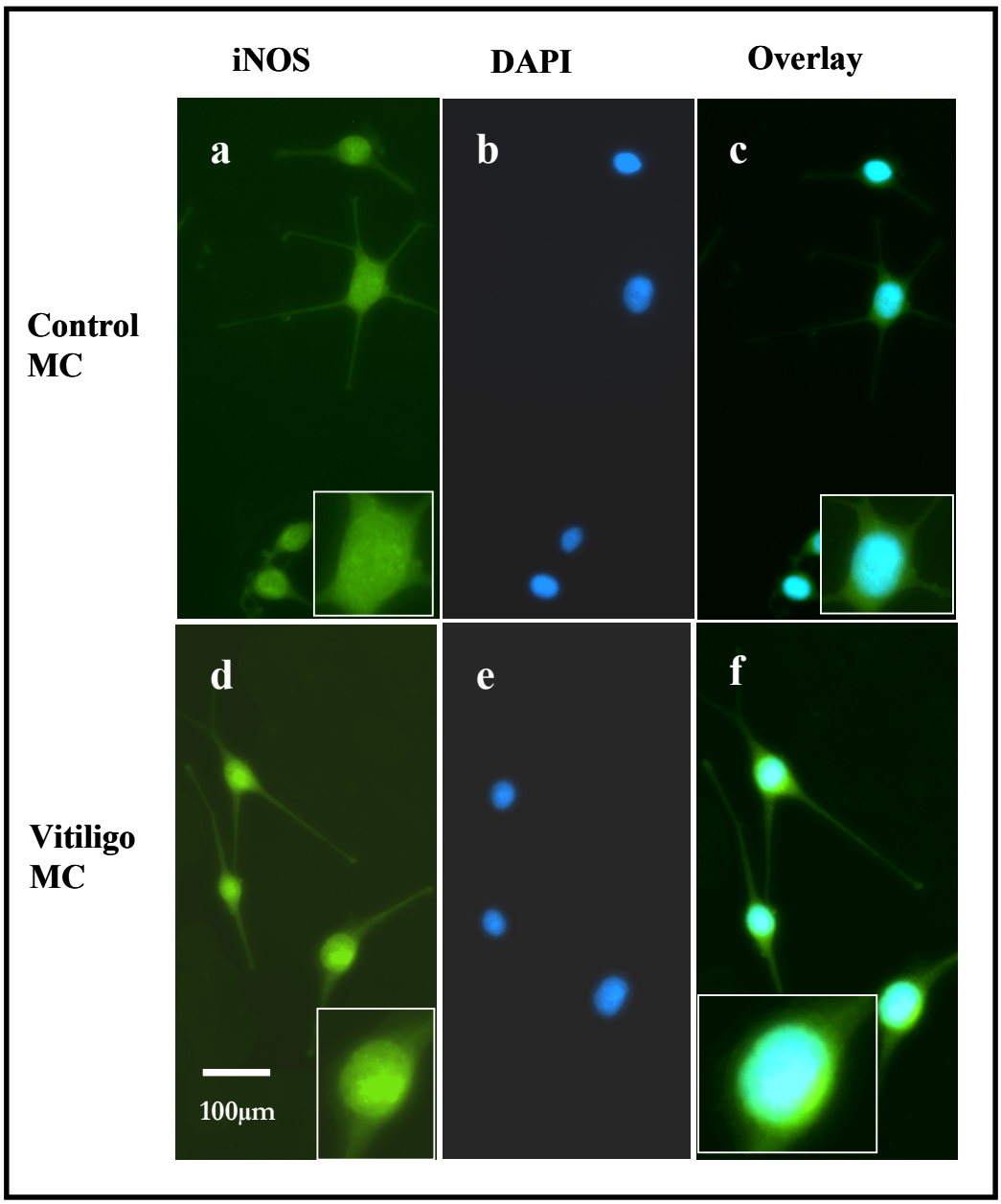


Figure 26
***In vitro* immuno-reactivity reveals up regulated iNOS in vitiligo melanocytes**
Melanocytes from patients with vitiligo are characterized by the presence of up-regulated nuclear iNOS (d and f respectively) when compared to healthy control cells (a and c respectively). (b) and (e) represent DAPI for control and vitiligo melanocytes respectively. Magnification x 400, scale bar 100μm

4.3.4 Up-regulated iNOS correlates with high expression of nitrated tyrosine residues in vitiligo

As said before, the observed increased levels of epidermal iNOS protein expression could contribute to a marked generation for NO in vitiligo. In this context it is important to mention that $O_2^{\cdot -}$ reacts with NO to give peroxynitrite ($ONOO^-$). The main footprint for the presence of $ONOO^-$ is nitrated tyrosine formed by the nitration of L-tyrosine in proteins. To define the formation of $ONOO^-$ in vitiligo, we monitored the rate of formation of nitro-tyrosine by using again immuno fluorescence staining with a specific antibody against nitrated tyrosine residues. As done in all other studies full skin biopsies from normal healthy control, (n=4) vitiligo lesional, (n=10) and vitiligo non lesional skin, (n=10) skin phototype III (Fitzpatrick classification) were used. The results show a pronounced *in situ* expression of nitrated tyrosine throughout the epidermis of patients with vitiligo lesional, non lesional skin compared to healthy controls **(Figure 27A)**. Quantification analysis of nitrated tyrosine expression reveals statistically significant increased nitrated tyrosine in the entire epidermis of vitiligo lesional skin (*** $p<0.001$, mean ± SD) vitiligo non lesional skin (*** $p<0.001$, mean ± SD) compared to controls **(Figure 27B)**. However, again there is no significant difference in the expression level of nitrated tyrosine in both epidermal vitiligo lesional and non lesional ($p>0.05$, mean ± SD).

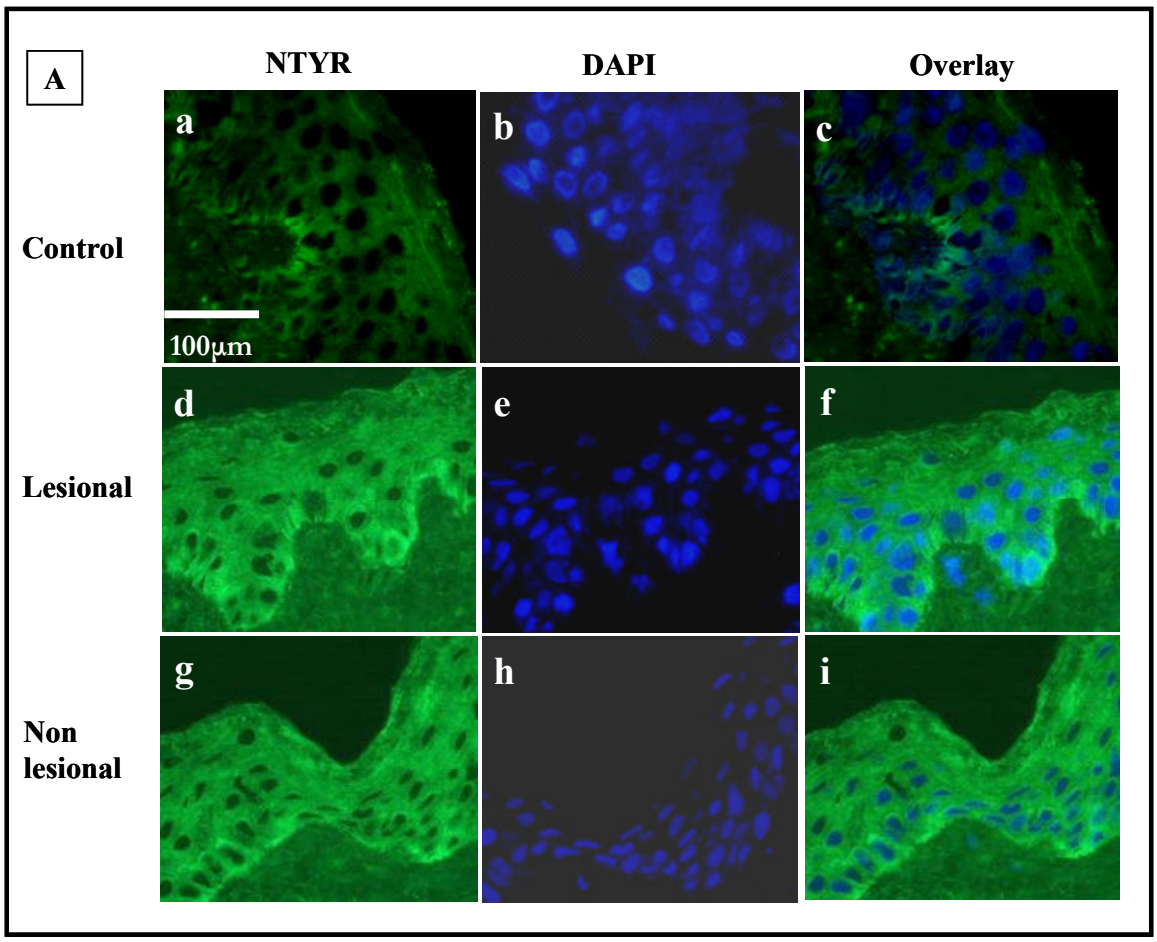
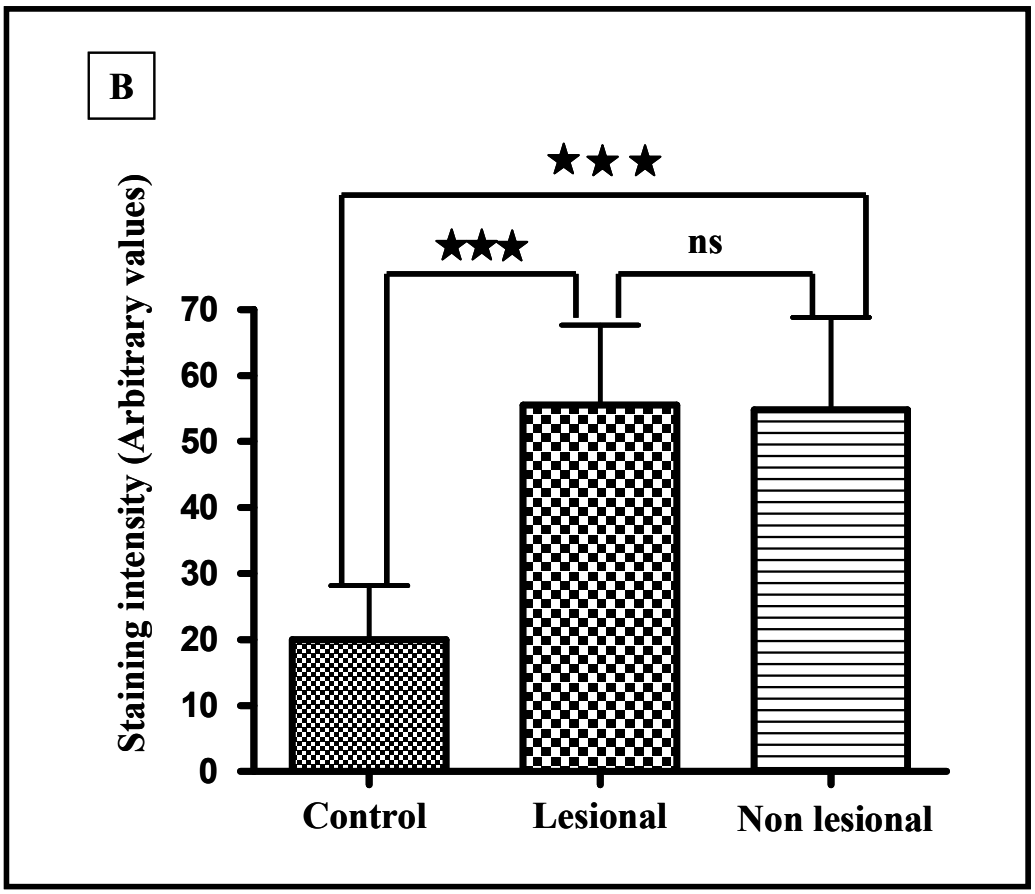


Figure 27
Significantly higher epidermal nitro tyrosine levels in vitiligo
(A) Immuno-reactivity staining (FITC labelling green) shows strong expression of nitrated tyrosine in vitiligo lesional (n=10) (d), vitiligo non lesional skin (n=10) (g), compared to healthy controls (n=4) (a), skin phototype III (Fitzpatrick classification) at magnification x 400. (b), (e) and (h) represent DAPI for healthy control, vitiligo lesional and vitiligo non lesional respectively. Nitrated tyrosine is not present in the nucleus. Scale bar 100µm.



(B) Image analysis Average fluorescence intensity shows significantly increased levels of nitrated tyrosine in both vitiligo lesional and non lesional skin compared to healthy controls. However, there is no significant difference between vitiligo lesional and non lesional skin. (*** $p<0.001$).

4.3.5 Increased *in vitro* expression of nitrated tyrosine in vitiliginous melanocytes

Epidermal melanocyte cell cultures established from patients with vitiligo and controls cells as mentioned in methods were used for immuno fluorescence staining. The result is consistent with the *in situ* data. Nitrated tyrosine residues are over expressed in vitiliginous melanocytes compared to control **(Figure 28d and a** respectively**)**. Moreover, the overlay with DAPI showed localization of nitro-tyrosine in the nucleus of vitiliginous melanocytes **(Figure 28f)**.

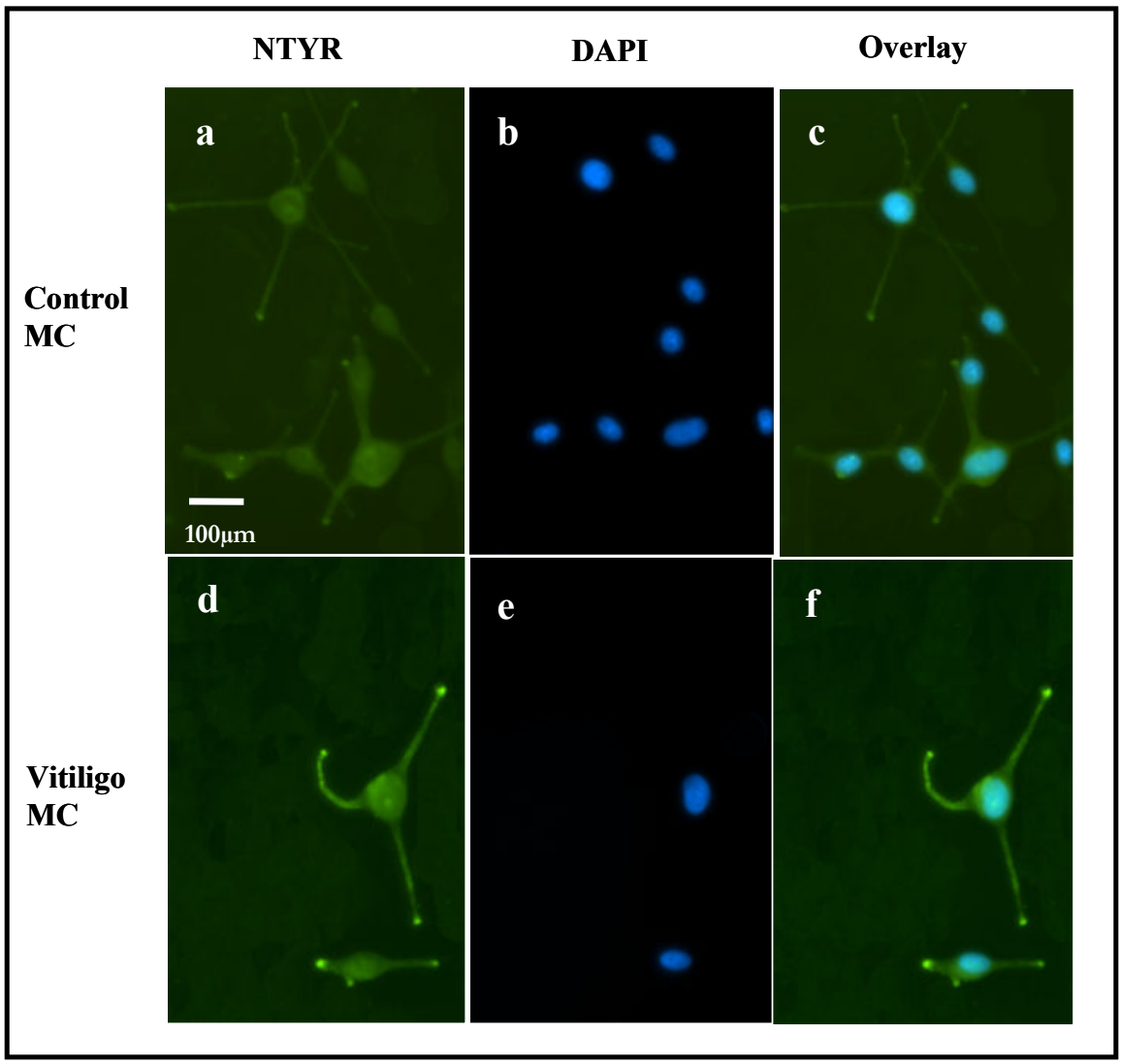


Figure 28
Vitiliginous melanocytes exhibit high levels of nitro tyrosine in the cytosol and in the nucleus
In vitro immuno fluorescence for vitiliginous and control melanocytes (d and a) respectively. DAPI for control and vitiliginous melanocytes is presented (b) and (e) respectively. The overlay shows nuclear localization of nitro-tyrosine in melanocytes from patients with vitiligo (f) compared to control melanocytes (c). Magnification x 400, scale bar 100µm.

4.4 Detection of nitrated p53 in vitiligo

4.4.1 *In situ* immuno-reactivity reveals signaficantly increased nitrated p53 expression in vitiligo skin

In situ and *in vitro* immuno fluorescence as well as Western blot revealed iNOS expression which in turn produces NO. In the presence of $O_2^{\cdot-}$, $ONOO^-$ is formed causing nitration of L-tyrosine yielding nitrated tyrosine. The levels of nitrated tyrosine are significantly higher and lesional and non lesional skin of the patients compared to controls (**Figure 29A**). Given that, p53 is up-regulated in vitiligo, we postulated that p53 nitration is taking place. In order to substantiate this assumption, we utilized, immuno fluorescence and Western blot. The results in **Figure 29A** revealed the presence of nitrated p53 in vitiligo lesional and non lesional skin as identified by a strong yellow colour after overlay of p53 and nitro-tyrosine expression (f and i respectively). Quantitative image analysis **(Figure 29B)** yielded a significantly higher expression in vitiligo lesional and non lesional skin compared to controls (*** $p<0.001$, mean ± SD). Notably, there was no significant difference between vitiligo lesional and non lesional skin.

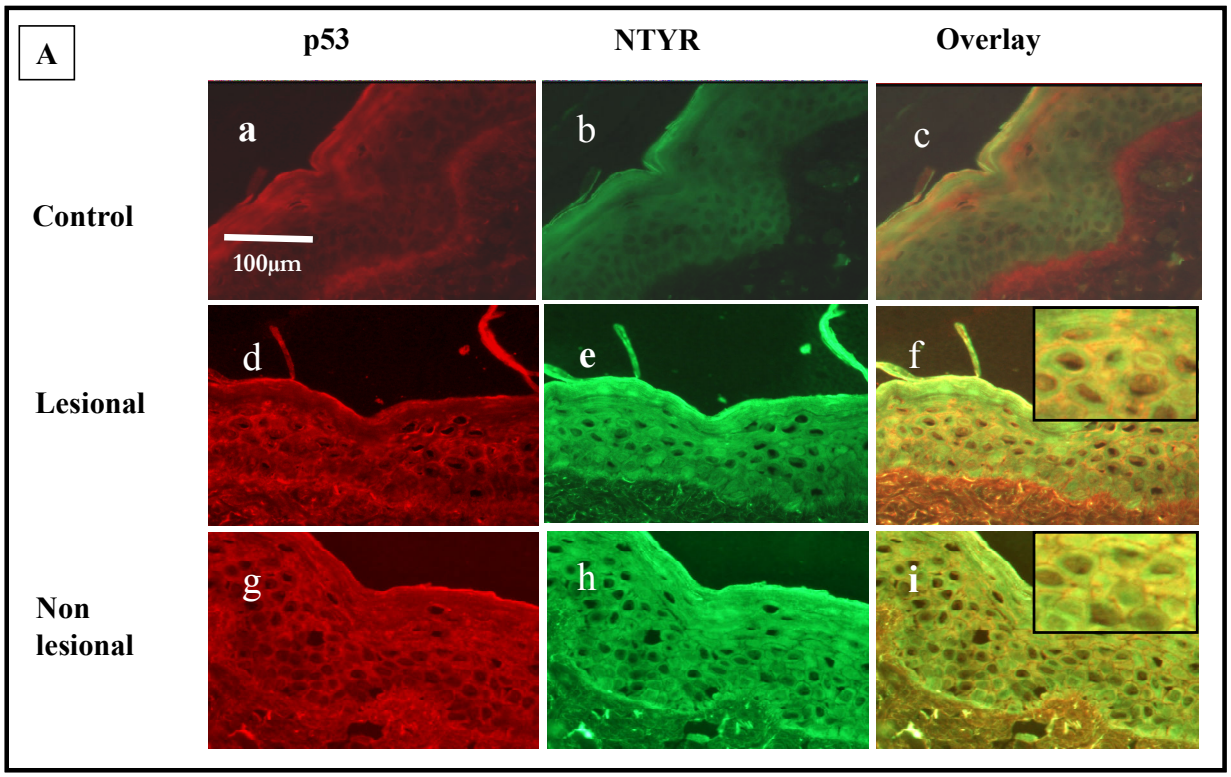
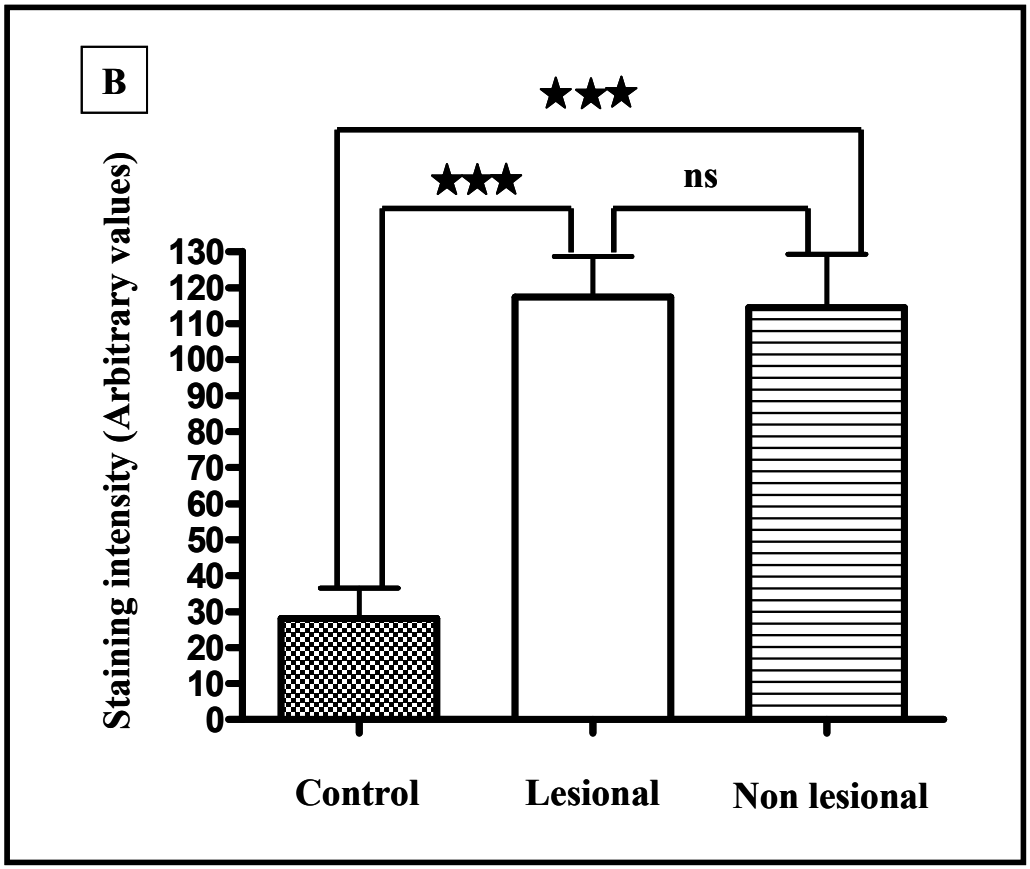


Figure 29.
Increased *in situ* expression of nitrated p53 in vitiligo
(A) Immuno fluorescence reactivity of TRITC-labelled p53 and FITC-labelled nitrate tyrosine (NTYR). (e and h) represent nitrated tyrosine in vitiligo lesional, (n=10) and non lesional skin, (n=10). Overlay of p53 and nitrated tyrosine identifies the presence of cytosolic nitrated p53 through our epidermis, spearing nuclei in (f) and (i), vitiligo lesional and non lesional skin respectively when compared to controls (c) (skin phototype III, Fitzpatrick classification). Magnification x 400, scale bar 100µm.



(B) Image analysis of nitrated p53 content. Nitrated p53 levels are significantly increased in vitiligo lesional (n=10) (*** $p<0.001$), non lesional (n=10) (*** $p<0.001$) compared to healthy controls (n=4). N.B. There is no difference between lesional and non lesional skin.

4.4.2 Western blot supports *in situ* immuno-reactivity of high nitrated p53

To further substantiate the presence of nitrated p53 in vitiligo, we explored epidermal suction blister cell extracts from patients with vitiligo by Western blot analysis for detection of native p53 and nitrated tyrosine. The tyrosine residue was nitrated by peroxynitrite. The results in **Figure 30** (lanes 3-10) prove ability of peroxynitrite to induce nitration for p53 in vitiligo compared to the control (lane 2). The results proved that p53 is nitrated in vitiligo. The results reveal the presence of increased p53 expression in epidermal vitiligo cell extract in association with increased nitrated tyrosine levels. The levels are significantly higher in all samples from patients compared to healthy controls. Together with the *in situ* results we can conclude that epidermal p53 is nitrated in vitiligo

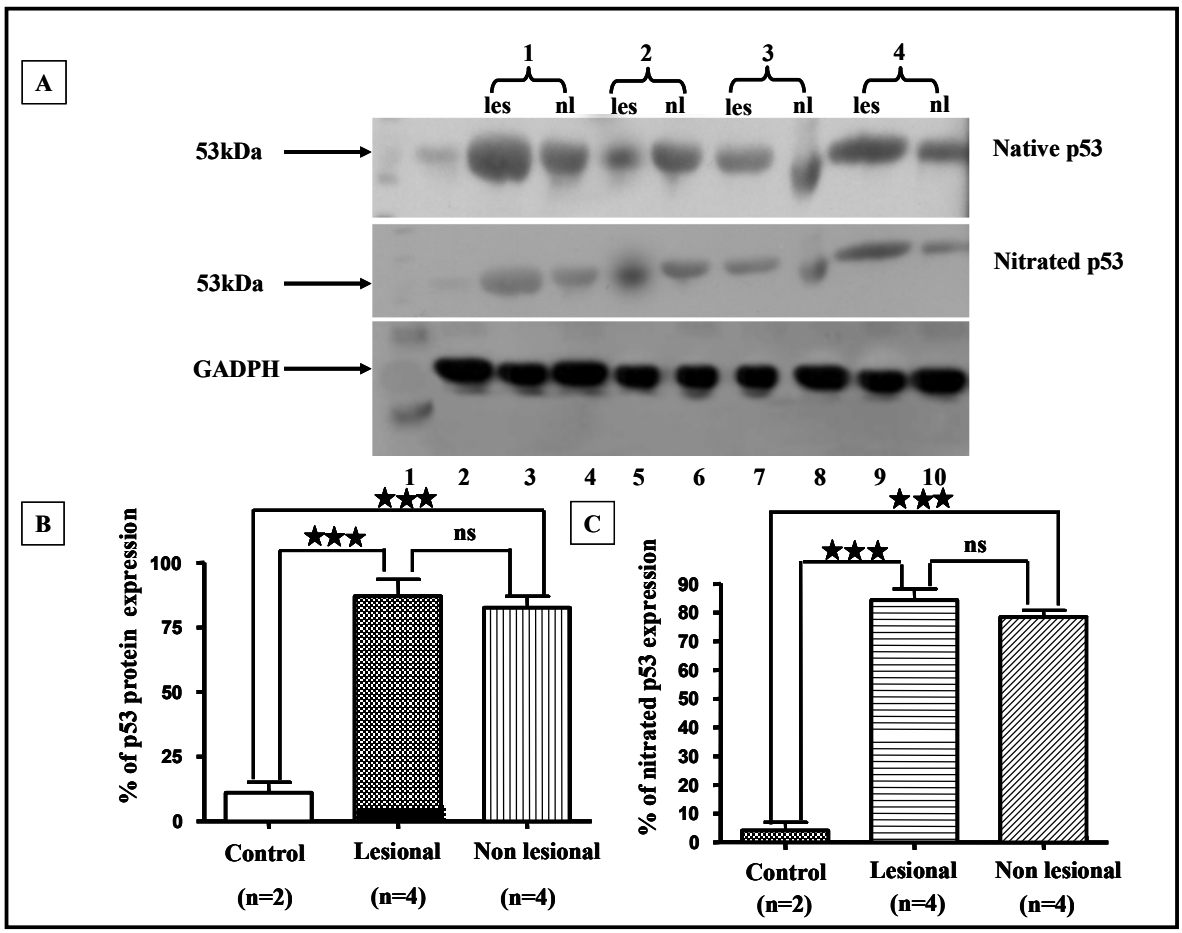


Figure 30
Accumulation of nitrated p53 in vitiligo
(A) Western blot analysis of total cell extracts from patients with vitiligo (n=4) (lanes 3-10) and healthy control (n=1) (lane 2). The results show strong p53 expression and association with strong N-tyrosine expression in vitiligo (lanes 3-10) compared to control (lane 2). Lane 1 represents protein ladder. GADPH was used as loading control.
(B) Image analysis of native p53 expression.
(C) Image analysis of nitrated p53 expression. Results show the significantly over expression of nitrated p53 in vitiligo compared to control. (Plots are mean ±SD). (*** $p<0.001$).

4.5 Expression of human p53 protein in *E. Coli*

At this point it was important to investigate our findings from the patients in more details. For this purpose we needed purified human p53 protein. *E. Coli* is the most important organism to be used to express p53, due to its long history of laboratory culture and ease of manipulation. Moreover, it is considered a very versatile host for the production of recombinant proteins.

4.5.1 Pilot experiment for determination of the optimal time for p53 protein expression from the BL21/DE3 containing the pT7.7 Hup53 construct

A preliminary time course experiment was performed to establish the optimum time for p53 expression in the T7 expression system as described previously in the Materials and Methods. In **Figure 31A** bands corresponding to p53 were neither observed in lane 2 (induced BL21/DE3 pT7.7Hup53), nor lane 3 (non induced BL21/DE3 pT7.7Hup53) as these samples were at time zero, they were also not observed in lanes 4, 5, 8 and 9 because these samples represented controls where the strain did not contain the *p53* gene. After 1 hour of induction by IPTG, a band of approximately 53 kDa was observed in lane 6, but it was not present in the non-induced control, lane 7. A p53 band was recognized in lane 2, (BL21/DE3 pT7.7Hup53), in lane 6 (BL21/DE3 pT7.7Hup53) **(Figure 31B)** and in lane 2 (BL21/DE3 pT7.7Hup53) **(Figure 31C)**. This band increased with intensity over time due to induction by IPTG after 2 hours and 3 hours respectively **(Figure 31B)** and 4 hours **(Figure 31C)**.

We also observed some induction in non induced cells as shown in lane 3 (BL21/DE3 pT7.7Hup53) and lane 7 (BL21/DE3 pT7.7Hup53) of **Figure 31B** and in

lane 3 (BL21/DE3 pT7.7Hup53) of **Figure 31C**. A band possibly corresponding to p53 protein was obtained. The intensity of this band increased over time. The reason for the possible expression of p53 protein in these non-induced cells is likely due to leaky expression of the *lac* promoter that promotes the transcription of *T7 RNA* polymerase gene even during non-induced IPTG conditions (Dubendoff and Studier, 1991).

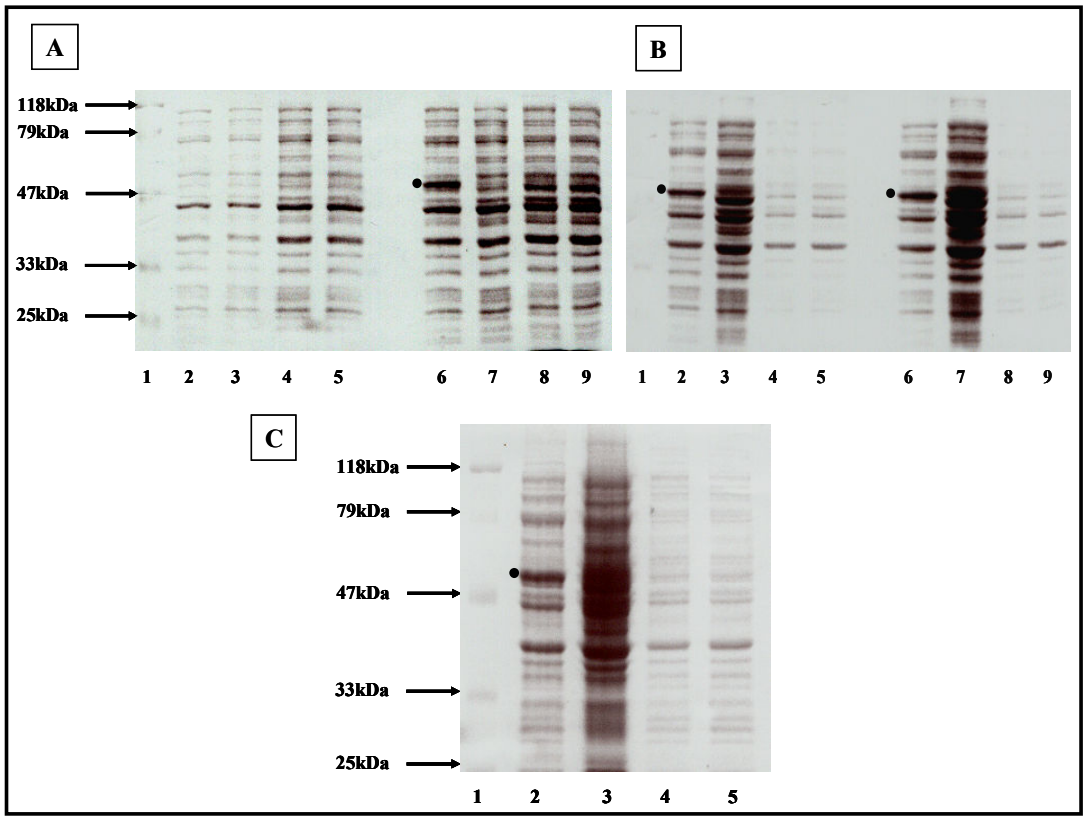


Figure 31
4 hours are the optimum period for the expression of p53 from *E. Coli*
(A) SDS-PAGE gel showing expression of p53 from E.*Coli* at time 0. There is no corresponding band for p53 in both lane 2 (induced, BL21/DE3 pT7.7Hup53) and lane 3 (non induced, BL21/DE3 pT7.7Hup53). Lane 4 and 5 represent induced and non induced BL21/DE3 respectively which do not contain p53 gene and act as negative controls. Lane 6 shows the expression of p53 protein by *E. Coli* after 1 hour induction by 1 mM IPTG when compared to lane 7 which represents non induced BL21/DE3 pT7.7Hup53. While lane 8 and 9 are negative controls strain (induced BL21/DE3 and non induced BL21/DE3) respectively. Lane 1 is the prestained protein marker. The dot indicates the position of p53 protein in SDS-PAGE gel.
(B) Lane 2 and 6 represent induced BL21/DE3 pT7.7Hup53 showing the expression of p53 protein by *E. Coli* after 2 and 3 hours induction by 1 mM IPTG respectively when compared to lane 3 and 7 which represent non induced BL21/DE3 pT7.7Hup53 during the same induction periods respectively. Lanes 4 and 5 represent negative controls strains (induced BL21/DE3 and non induced BL21/DE3) respectively after 2 hours induction. Lane 8 and 9 are the same negative controls strain but after 3 hours induction. Lane 1 is the prestained protein marker. The dot indicates the position of p53 protein in SDS-PAGE gel.
(C) Lane 2 (induced BL21/DE3 pT7.7Hup53) demonstrates the maximum expression of p53 from *E. Coli* after 4 hours induction. Lane 3 represents non induced BL21/DE3 pT7.7Hup53. While lane 4 and 5 are negative controls strain (induced BL21/DE3 and non induced BL21/DE3) respectively which do not contain p53 gene. Lane 1 is the prestained protein marker. The dot marks the position of p53 protein in SDS-PAGE gel.

4.5.2 Measurement of the optical density for all the *E. Coli* strains used for p53 expression

In order to investigate the growth rate in all strains used for p53 expression, the optical density of OD_{650nm} of the induced strains and non-induced was measured prior to sample preparation for SDS-PAGE gel **(Figure 32)**. The retarded growth in the BL21/DE3 pT7.7Hup53 was not observed in the host BL21/DE3 strain as expected (Studier *et al*., 1990), reflecting the effect of over expression from the powerful *T7* promoter.

It was observed from the SDS-PAGE gel that the BL21/DE3 pT7.7Hup53 strain after induction exhibited a weaker protein stain profile than the non-induced strain reflecting the retardation of growth as shown **(Figure 32)**.

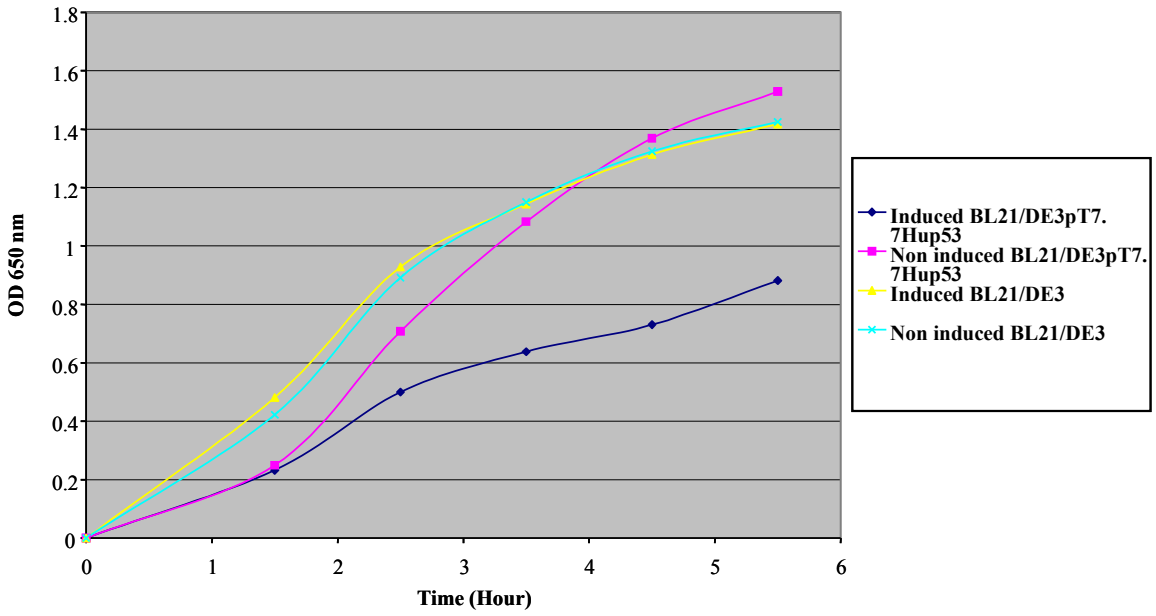


Figure 32
Growth curves for the strains used for p53 expression
Upon addition of IPTG the growth of all bacterial strains started after 90 minutes (BL21/DE3 pT7.7Hup53 and BL21/DE3). In the absence of IPTG no growth was detected. The induction with 1 mM IPTG was performed every 60 min over 4 hours.

4.5.3 Confirmation of p53 expression from *E. Coli* by Western blotting

Following the pilot expression of human p53, Western blots were carried out for further confirmation. Aspecific antibody for p53 (NCL-p53-DO-1, Novacastra, UK) was used. The results demonstrate in lanes 2 and 4 of **Figures 33A** and **B** a strong band, corresponding to p53 protein, and this band revealed an increase in intensity with the time of induction, 1, 2, 3 and 4 hours respectively by 1 mM IPTG. It is noticeable that the induced samples in lanes 2 and 4 of **Figures 33A** and **B** showed a smear protein below the full molecular size of p53 protein. This result may be due to the extensive sonication used to prepare the samples for SDS-PAGE analysis. Moreover, a weak band for p53 protein in non-induced cells was detected in lanes 3 and 5 of **Figures 33A** and **B**. This band was most likely due to the known leaky activity of *lac* promoter (Dubendoff and Studier, 1991).

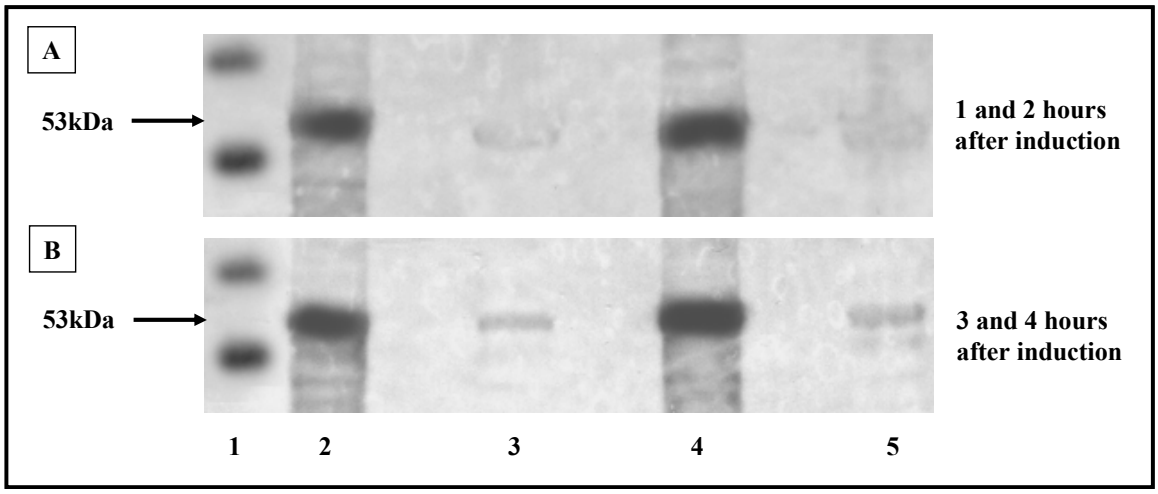


Figure 33
Human p53 protein expression from *E. Coli* strain (BL21/DE3 pT7.7 Hup53) after induction with 1 mM IPTG over time (1, 2, 3 & 4 hours)
(A) Western blot analyses the detection of p53 protein after induction with 1 mM IPTG by 1 and 2 hours using *E. Coli* strain, BL21/DE3 pT7.7 Hup53, Lane 2 and 4 respectively. Lanes 3 and 5 represent non induced BL21/DE3 pT7.7 Hup53. Protein ladder is represented in lane 1.
(B) Increased intensity of p53 protein expression after induction by 3 and 4 hours (lanes 2 and 4). Lanes 3 and 5 (non induced BL21/DE3 pT7.7 Hup53) exhibit p53 protein expression due the leaky activity of *lac* promoter. Lane 1 is protein ladder.

4.5.4 Large scale expression of human p53 from BL21/DE3 pT7.7 Hup53

After determination of the optimum time (4 hours) for p53 expression from *E. Coli* strain BL21/DE3 pT7.7 Hup53. A large scale of p53 expression was carried out by scaling up to 2 litres. The results revealed increased expression of p53 protein in a time dependent manner after induction with 1 mM IPTG. There is no expression for p53 protein before induction as shown in lane 2 **(Figure 34)**.

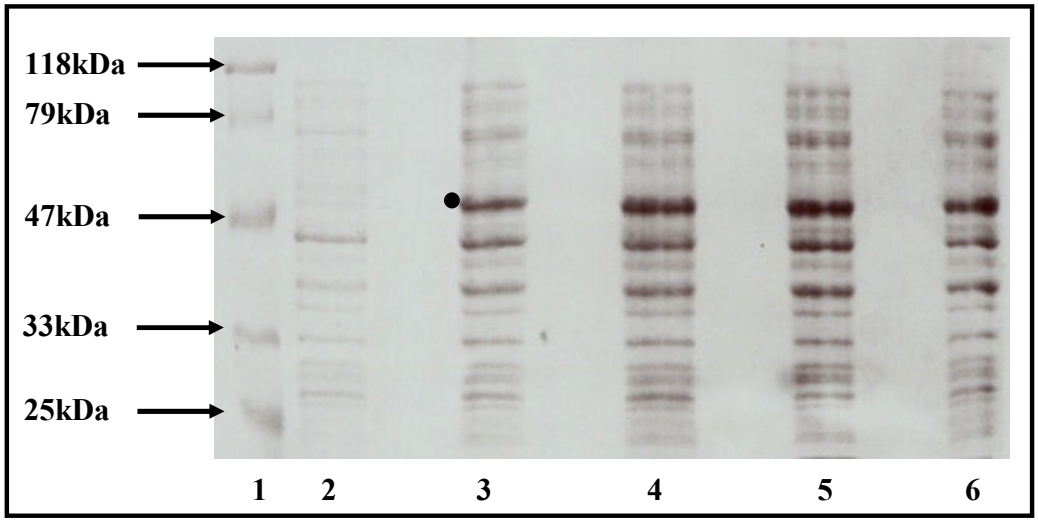


Figure 34
p53 protein expression from *E. Coli* BL21/DE3 pT7.7 Hup53, before and after induction with 1 mM IPTG
SDS-PAGE gel showing the expression of p53 protein throughout the induction time, 1, 2, 3 and 4 hours (lanes 3, 4, 5 and 6 respectively) marked by dot. There is no expression for p53 before induction, lane 2. Lane 1, prestained protein marker.

4.6 Nitration of the resolubilised human p53 protein by peroxynitrite

Our data showed that p53 undergoes nitration in vitiligo. In order to substantiate these findings, we utilized resolubilised purified p53 from *E. Coli.*

4.6.1 Peroxynitrite nitrates the resolubilised p53 protein

A pilot experiment for nitration of resolubilised p53 protein was performed by treating the protein with different concentrations of PN (0.1 mM and 0.5 mM) using nitrated BSA (0.5 mM of PN) as a positive control. The results of the nitration experiment show a strong band which is in agreement with nitrated BSA protein **(Figure 35A)**. This result proves PN acting as a nitrating agent for tyrosine residues in p53 protein confirming earlier data in the literature (Berlett, Friguet et al. 1996).

4.6.2 Dose response for nitration of p53 by peroxynitrite

To gain further insight into the process of nitration on p53 protein, we investigated the effect of different PN concentrations (20 μM, 40 μM, 60 μM, 80 μM and 100 μM) on the resolubilised p53 protein. Nitrated BSA (50 μM of PN) was used as a positive control. Our result revealed that nitration of p53 is a dose dependent **(Figure 35B)**.

4.6.3 Nitration of p53 by peroxynitrite is time dependent

Next we investigated the effect of time on nitration of p53. In this experiment the following time intervals (1 sec, 5 sec, 15 sec, 30 sec, 60 sec and 90 sec) were used to follow nitration of p53 protein in the presence of 0.1 mM PN. The results showed an increase in the formation of nitrated p53 protein up to 30 sec **(Figure 35C)**, while time > 30 sec had no additional effect. Nitrated p53 (0.1 mM PN) was used as a positive control **(Figure 35C)**. From this result we can conclude that nitration of tyrosine residues is a fast event, taking place in seconds.

Taken together, our *in vitro* results using purified human p53 confirmed nitration of p53 as shown by Berlett and colleagues in 1996. We show that this process is fast and dependent on the presence of PN concentration.

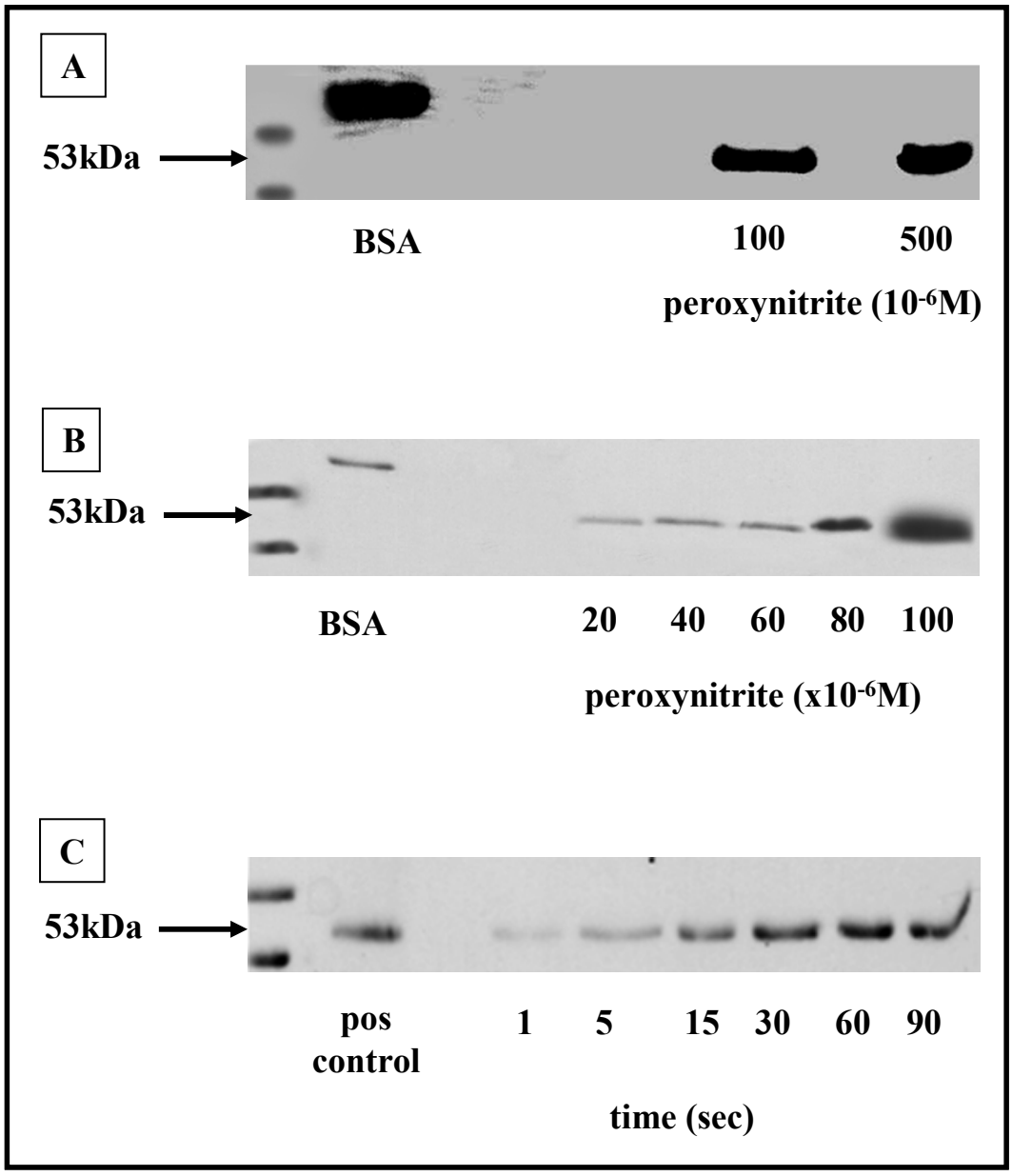


Figure 35
Nitration of the resolubilised p53 by PN is dose and time dependent
(A) The resolubilised p53 was nitrated with concentrations of 100 and 500 x10^{-6}M of PN. Nitrated BSA protein was used as a positive control.
(B) Nitration of p53 is concentration dependent of PN, (20-100 μM). Nitrated BSA (50 μM) served as a positive control.
(C) Nitration of p53 by PN is time dependent. NB gradual increases up to 30 sec. Exposure >30 seconds had no additional effect. Positive control, nitrated p53 (0.1 mM PN).

4.7 Nitration of immunoprecipitated p53 protein from HT-29 cells

To further explore the effect of $ONOO^-$ on p53 protein, we immunoprecipitated p53 protein from HT-29 cells by using protein G agarose beads and antibodies specific for p53 as mentioned earlier in materials and methods (section, 3.8). The HT-29 cell line obtained from human colon carcinoma and was chosen for this study because it contains a mutant p53 protein. Here it was of interest whether PN had the ability to nitrate mutant p53.

4.7.1 Immunoprecipitation of p53 protein from HT-29 cells

In order to prove nitration of mutant protein, we prepared a large amount of mutant p53 protein from HT-29 cells. Protein G agarose was used for immunoprecipitation because it has a high affinity for IgG, e.g. DO-1. **Figure 36A** shows a condensed band in the position of immunoprecipitated mutant p53 protein in lane 10. To further confirm immunoprecipitation of p53 protein, a positive control has been used (resolubilised p53 protein from *E. coli* strain, BL21/DE3 pT7.7Hup53, lane 2). Lane 3 represents HT-29 cell extract serving as a control for estimating the amount of mutant type p53 protein in proportional to the resolubilised p53 protein (lane 2). Lanes 4, 5, and 6 represent controls for protein G agarose including protein G agarose only, protein G agarose+HT-29 cell extracts after washing with HSB and protein G agarose+TH-29 cell extract after washing with HSB for the second time respectively. This step done to show that protein G agarose can not bind to mutant type of p53 protein after incubation together in the absence of the antibody (DO-1). Lanes 7 and 8 represent the loading of clearing supernatant and the washing solution

of the immunocomplex (protein G agarose+DO-1+mutant type of p53 protein) with HSB respectively. These two lanes show a band corresponding to mutant type p53 protein. This result was acceptable because the DO-1 antibody cannot bind to all mutant type p53 protein molecules. This is also the case for protein G which cannot bind to the entire immunocomplex. In lane 9 there is not any band for mutant p53 protein because it represents the washing solution of the immunocomplex with HSB for the second time. This result confirms that there was no non-specific binding. To provide more confirmation the same sample as in lane 10 **(Figure 36A)** was subjected to further analysis by Western blot. This data **(Figure 36B)** revealed that mutant p53 protein was clearly detected by the immunoprecipitation obtained with protein G agarose and DO-1 antibody **(Figure 36A)**. The data in **Figure 36B** show detection of different amounts of mutant p53 protein from HT-29 cells. Lanes 2, 3, 4, 5, and 6 represent bands for mutant p53 protein bands corresponding in a concentration dependent manner (2 µl, 4 µl, 6 µl, 15 µl and 20 µl of p53 protein).

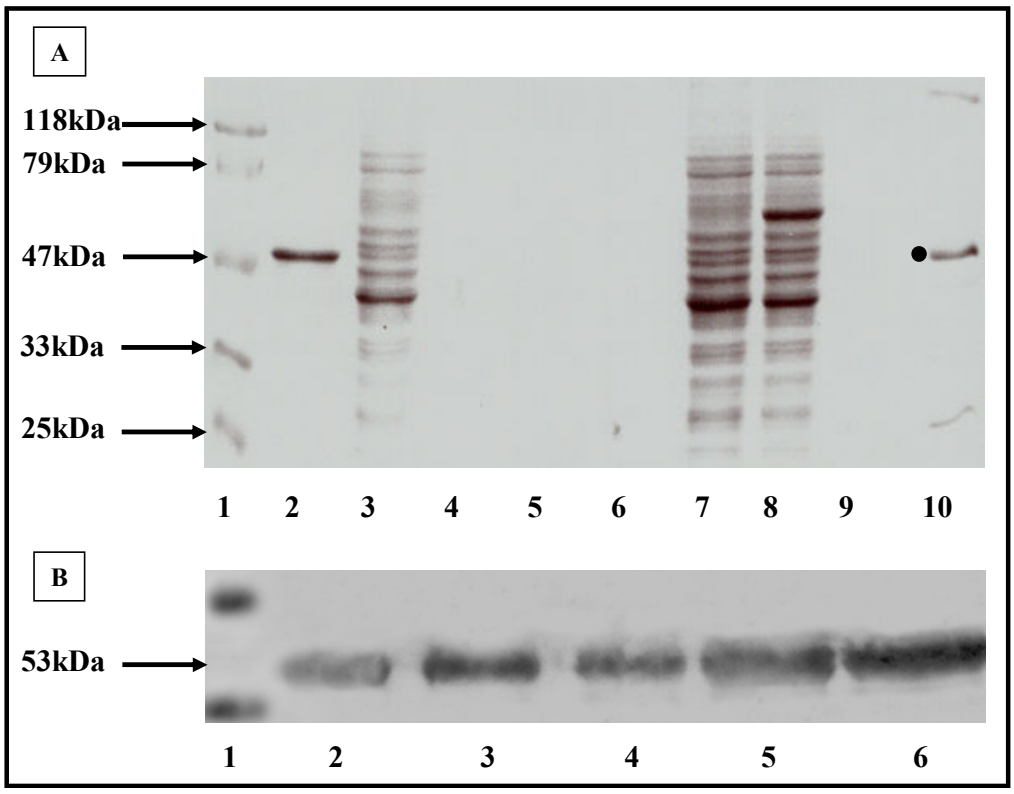


Figure 36
Immunoprecipitation of mutated p53 protein
(A) SDS-PAGE showing the successful immunoprecipitation for p53 protein from HT-29 cells. Lane 1 is the prestained protein marker. Lanes 2 and 3 are representing positive control and total cell extract from HT-29 cells respectively. Lanes 4, 5 and 6 are serving as protein G agarose only, protein G agarose+HT-29 cell extract after washing with HSB and protein G agarose+TH-29 cell extract after washing with HSB for the second time respectively. The loading of supernatant, the washing solution of the immunocomplex (protein G agarose+DO-1+mutant type of p53 protein) with HSB and the washing solution of the immunocomplex with HSB for the second time are shown in lanes 7, 8.and 9 respectively. The dot indicates the position of the immunoprecipitated p53 in lane 10. On the other hand the light and heavy chain for the DO-1 antibody is located above and below the p53 band.
(B) Western blot shows the detection of immunoprecipitated p53 (lanes 2-6) from HT-29 cells using CM-1 antibody (1:1000) after performing titration with different amounts of p53 (2 µl, 4 µl, 6 µl, 15 µl and 20 µl). Lane 1 serves as protein ladder.

4.7.2 Immunoprecipitation of the nitrated mutant p53 protein from HT-29 cells

The nitration process for mutant p53 protein (HT-29 cells) was followed by using the DO-7 labelled immunocomplex after nitration with 0.5 mM PN (protein G agarose+CM-1+ mutant p53 protein) to demonstrate that the immunoprecipitated protein was indeed mutant p53 protein. After stripping by using anti-nitro tyrosine antibody only one band was detected **(Figure 37B)**. From these results we can conclude that mutant p53 from HT-29 cells is still subjected to nitration.

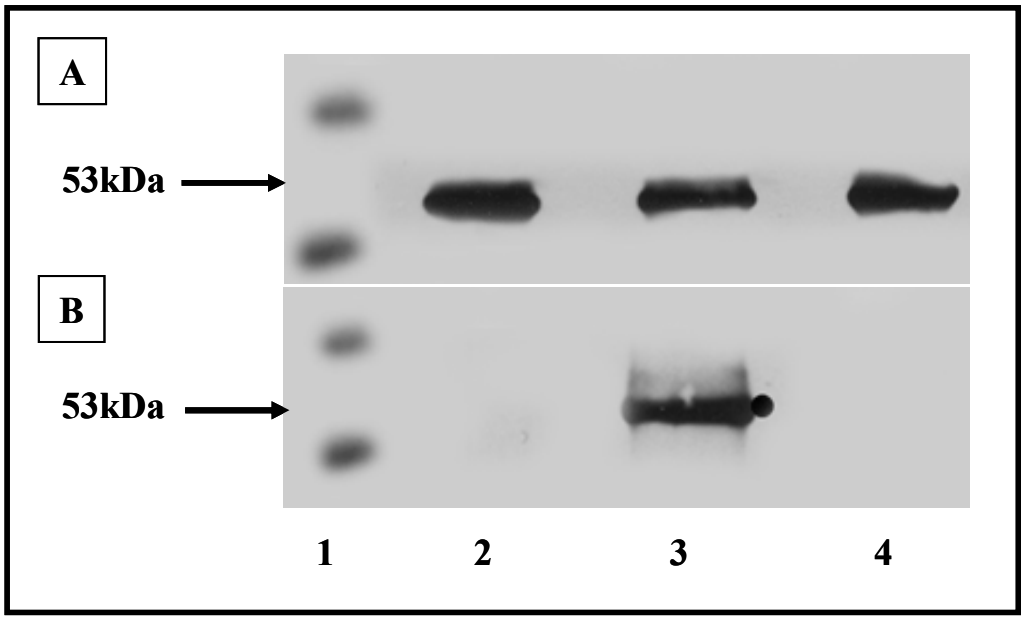


Figure 37
Detection of nitrated mutant p53 protein from HT-29 cells
(A) Western blot shows the detection of the immunoprecepitated p53 from HT-29 cells by using DO-1 antibody. Lane 2 represent pellets (HT-29 cells non nitrated+protein G agarose+CM-1), lane 3 pellets (HT-29 cells +protein G agarose + CM-1) nitrated with 0.5 mM PN and lane 4 pellets (HT-29 cells +protein G agarose + CM-1) decomposed 0.5 mM PN respectively.
(B) Stripping showing the identification of the immunoprecipitation of nitrated mutant p53 protein (HT-29 cells) lane 3. The primary antibody is anti-nitro tyrosine (1:1000) and the secondary antibody is rabbit anti mouse IgG HRP (1:1000). The dot indicates the position of nitrated p53 protein. Lane 1 is serving as protein ladder.

4.7.3 Dose response for nitration of mutant p53 protein

This experiment has been performed to investigate the effect of different concentrations of PN (10 μM, 50 μM, 100 μM and 250 μM), as well as the effect of the decomposed 0.5 mM PN on p53. The stripping data show that treatment of mutant p53 protein with different concentration of PN exhibits a dose response. There is a gradual increase in nitration of p53 protein proportional to the concentration of PN as shown in lanes 2, 3, 4 and 5 of **Figure 38A.** There was no effect for decomposed PN as shown in lane 6. Western blotting **(Figure 38B)** demonstrates nearly equal amounts of p53 protein in all wells after stripping supporting the result that mutant p53 can be nitrated.

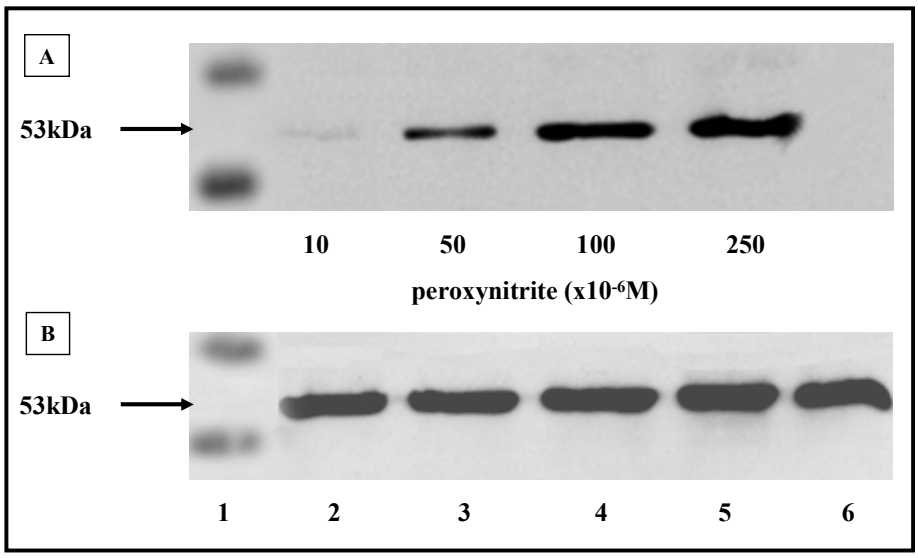


Figure 38
Nitration of mutant p53 protein is dose dependent
(A) Western blot shows the dose response for the nitrated immunoprecepitated p53 from HT-29 cells by using anti-nitro tyrosine (1:1000) antibody, specific for nitrated p53 protein at different concentration of PN (10 μM, 50 μM, 100 μM and 250 μM), lane 2, 3, 4 and 5 respectively. Lane 6 represents the decomposed PN.
(B) After **s**tripping showing the identification of the same mutant p53 protein levels (HT-29 cells) lane 2-6. The primary antibody was DO-1 (1:1000). Lane 1 represents protein ladder.

4.7.4 Effect of both nitration and oxidation on p53-DNA binding

The presence of high epidermal H_2O_2 levels in association with up-regulated nitrated p53 in the epidermis of patients with vitiligo calls into questions whether oxidation / nitration affect DNA-binding capacity. In order to get a better insight into this process we performed EMSA. The results revealed that after nitration of p53 protein its binding capacity to DNA oligonucleotide sequence is completely abrogated in the presence of >300 μM $ONOO^-$ **(Figure 39A)**. When p53 protein was subjected to oxidation by H_2O_2 using different concentrations (100 – 800x 10^{-6} M and 10^{-3} M) p53-DNA binding was improved **(Figure 39B)**. Importantly , after p53 protein was incubated for the maximum of 1 minute with combined H_2O_2 and $ONOO^-$ (1:1), the DNA binding capacity was further enhanced **(Figure 39C)**.

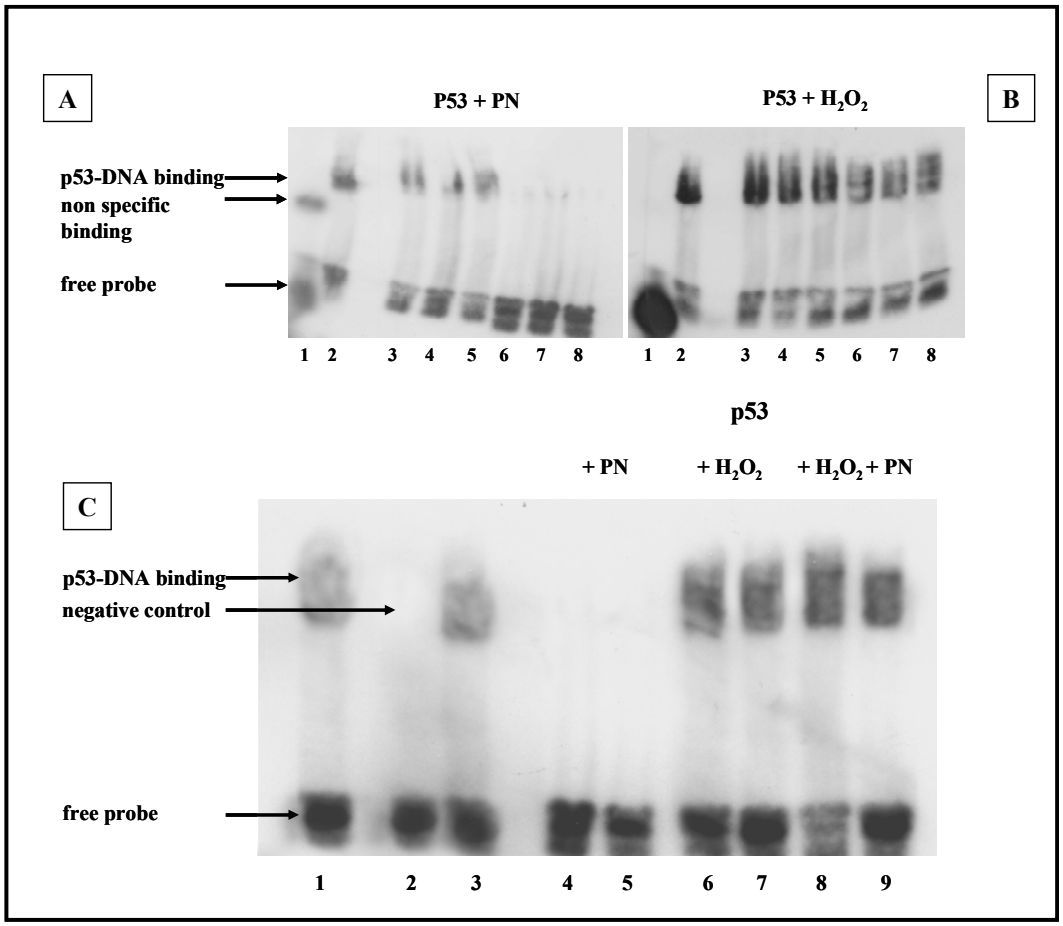


Figure 39

The effect of peroxynitrite-mediated nitration and H_2O_2-mediated oxidation and the contribution of both on DNA binding capacity of p53

(A) PN mediated nitration abrogates p53-DNA binding in a dose dependent manner (lanes 6-8) (500, 800 x 10^{-6} M and 10^{-3} M respectively). Low concentrations of PN, has no effect on DNA-binding (lanes 3-5) (100, 200, 300 x 10^{-6} M respectively). Lane 1 negative control, lane 2 native p53 (positive control).

(B) H_2O_2-mediated oxidation Increase p53-DNA binding capacity (lanes 3-8) (100, 200, 300, 500, 800 x 10^{-6} M and 10^{-3} M respectively). Lane 1 negative control, lane 2 native p53 (positive control),

(C) P53-DNA binding was improved as a result of combined effect of H_2O_2 and PN (lanes 8 and 9) (10^{-3} M) when compared to H_2O_2 effect only (lanes 6 and 7) (10^{-3} M). However lanes 4 and 5(10^{-3} M) represent the abrogation of p53-DNA binding due to the effect of PN. Lanes 1 and 3 positive controls, lane 2 negative control.

4.7.5 Computer simulation supports enhanced DNA binding of p53 due to combination of H_2O_2-mediated oxidation plus nitration

In the native structure of p53 there are seven amino acids (lys 120, ser 241, arg 248, arg 273, ala 276, cys 277 and arg 280) in the DNA binding site which bind 6 nucleotides (Cho et al 1994) **(Figure 40A)**. Nitration converts 8 tyrosine residues to 3-nitrotyrosine in the DNA binding domain of p53 (tyr 103, tyr 106, tyr 126, tyr 163, tyr 206, tyr 220, tyr 234, tyr 236) leading to severe changes in the secondary and tertiary structure. From the 7 residues involved in DNA binding (lys 120, ser 241, arg 248, arg 273, ala 276, cys 277 and arg 280) arg 248 and cys 277 are lost yielding a reduced nucleotide binding capacity **(Figure 40B)**. This result is consistent with our gel shift assay.

After applying oxidation by H_2O_2 the nucleotide, binding enhances due to the participation of 13 amino acids instead of 7. From the original complex lys 120, arg 248, arg 273, cys 277 and arg 280 are retained, whereas H-bonds from ser241 and ala 276 are lost. However, there are now 8 new amino acids (val 122, lys 139, his 179, ser 240, arg 249, pro 250, val 274, arg 283) H-bonding to DNA. This result supports a pronounced increase in DNA binding **(Figure 40C)**. Combined oxidation and nitration of met, trp and tyr residues results in a number of structural changes but most of the original amino acids remain, including those most crucial to p53-DNA binding. New H-bonding interactions are formed from 10 new amino acids, increasing the number of amino acids interacting to 15. However, the model expects the binding of 10 nucleotides in the oxidised complex **(Figure 40D)**. This work has been contributed by Dr NCJ Gibbons from our group.

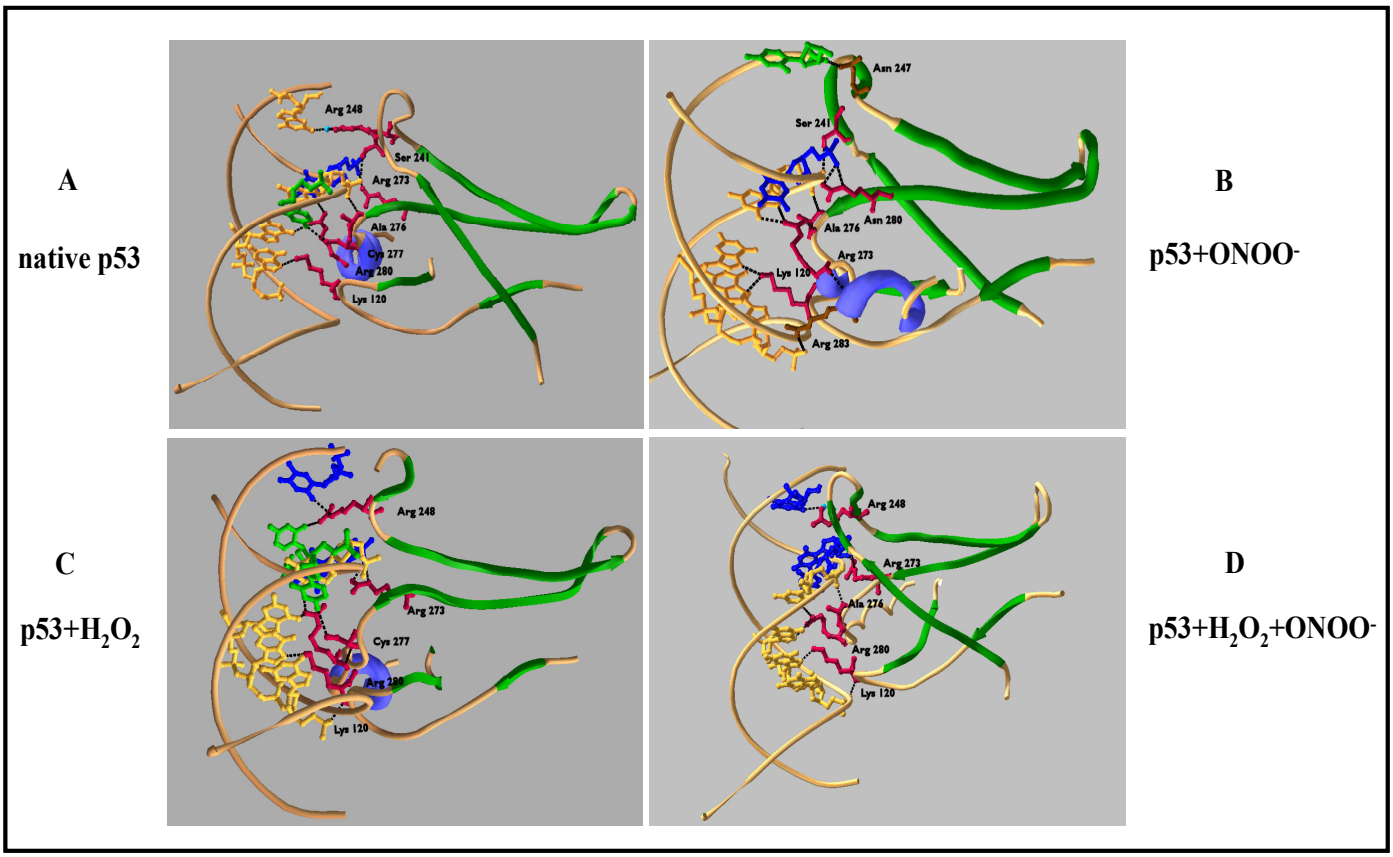


Figure 40
Computer modelling of p53 in the presence and absence of H_2O_2 and $ONOO^-$ after H_2O_2-mediated oxidation and after nitration / oxidation
(A) Native p53-DNA complex
DNA binding to p53 involves seven amino acids forming the basis for interaction to the DNA backbone and the major and minor grooves via H-bonds to 6 nucleotides.
(B) Effect of PN mediated nitration on DNA binding of p53
Nitration of tyrosine residues in p53 does not affect the overall number of residues interacting with DNA. However, two residues are lost and substituted by new amino acids, including one of the crucial arg residues, arg^{248}. This result supports the DNA binding data.
(C*)* H_2O_2-mediated-oxidation of the p53 -DNA binding site enhances nucleotide binding capacity
Oxidation of met and trp residues results in changes in the structure of p53 in association with the loss of some original amino acids. However, key amino acid residues remain H-bonded to DNA but there are new H-bondings from 8 new amino acids increasing the number of amino acids interacting with DNA to 13 in association with the binding of 10 nucleotides. This result is in support with the data obtained with oxidised p53 as shown by EMSA.
(D) Oxidation / nitration enhances DNA binding of p53
Oxidation of met, trp and tyr residues results in a number of structural changes. However, once again, most of the original amino acids remain, including those most crucial to the binding of p53 to DNA. In addition new H-bonding interactions are formed from 10 new amino acids, increasing the number of amino acids interacting to 15. The number of nucleotides bound is the same as with the oxidised complex.
Colour key–nucleotides, yellow–guanosine, blue–thymine, green–cytosine (no adenine nucleotides involved. Original amino acids–burgundy, new amino acids–brown. (With kind permission from Dr NCJ Gibbons)

4.8 Activation of p53 protein in vitiligo

Under normal physiological conditions p53 is regulated by mdm2. In response to genotoxic stress, such as DNA damage, p53 is hyperphosphorylated and acetylated at multiple sites within or near the mdm2 binding domain (Giaccia and Kastan 1998; Chehab, Malikzay et al. 1999). It was shown by several groups (Hirao, Kong et al. 2000; Shieh, Ahn et al. 2000) that DNA damage causes activation of ATM kinase promoting phosphorylation and acetylation of p53 which in turn leads to its stabilization by preventing mdm2 from binding to p53 (Shieh, Ikeda et al. 1997); (Unger, Juven-Gershon et al. 1999).

4.8.1 Evidence for phosphorylation of p53 in vitiligo

Phosphorylation is an important step for stabilization and activation of p53 in order to perform its function as a transcription factor (reviewed in Giaccia and Kastan 1998; Appella and Anderson 2000). In this context it was shown that this process is induced by many stress factors including DNA damage affecting mainly 30 amino acids residues at both the amino terminus and the carboxyl terminus (Appella and Anderson 2001). Cellular stress activates the expression of a protein kinase family including ATM and ATR which mediate phosphorylation of ser 15, ser 9 and ser 37 on p53 (Canman, Lim et al. 1998; Tibbetts, Brumbaugh et al. 1999).

4.8.1.1 Epidermal ATM is significantly increased in vitiligo

To follow ATM protein expression, epidermal suction blister cell extracts were analyzed by Western blot **(Figure 41).** The results revealed that ATM expression is significantly higher in cell extracts from patients with vitiligo (lane 3-12) while in healthy controls ATM is barely detectable (lane 2). These results suggest that the increased p53 protein in vitiligo is phosphorylated / activated by ATM due to the presence of DNA damage as pended down by the presence of 8xoG levels in the epidermis and plasma of patients with vitiligo (Shalbaf 2009).

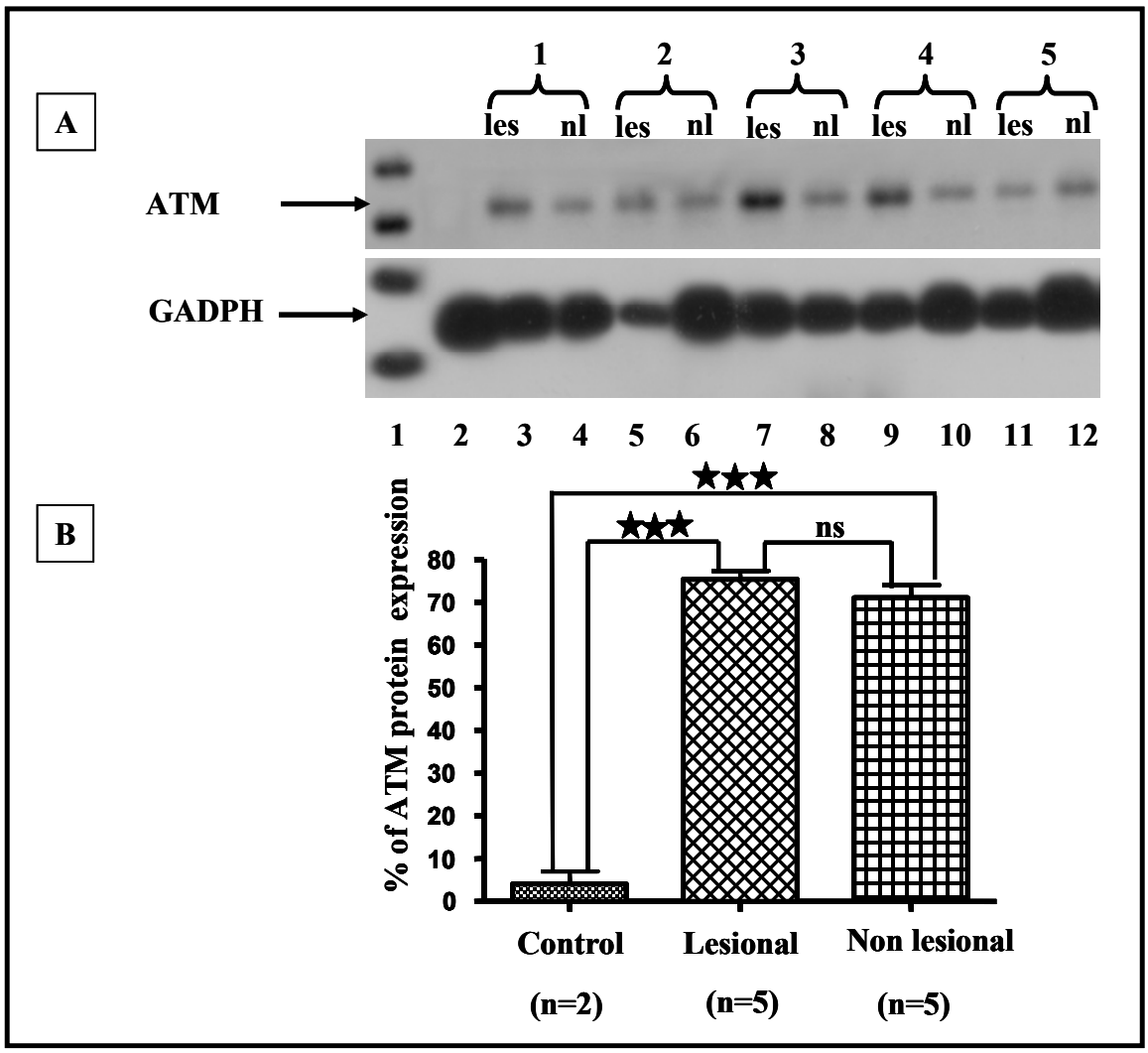


Figure 41
Epidermal cell extracts from vitiligo reveal induced ATM protein expression
(A) Western blot analysis shows an increase of ATM protein expression in all 5 patients with vitiligo tested (lanes 3-12) while ATM is absent in healthy control (lane 2), lane 1 protein ladder. GADPH was used as a loading control.
(B) Densitometry analysis of the band related to GADPH. ATM protein expression is significantly higher in vitiligo lesional compared to control (plots are mean ± SD). (*** $p<0.001$).

4.8.1.2 Expression of ATM in vitiligo melanocytes

Briefly, ataxia telangiectasia mutated (ATM) is a serine/threonine-specific protein kinase. The protein is named based on the disorder Ataxia telangiectasia caused by mutations of ATM. This protein is recruited and activated by DNA damage. It phosphorylates several proteins that initiate activation of DNA damage checkpoints, leading in turn to cell cycle arrest, DNA repair or apoptosis. One of these targets is p53.

In this thesis we showed the presence of nitrated p53 in patients with vitiligo together with up-regulated p53. In this contest it is noteworthy that earlier data from our group demonstrated up-regulated functional p53 (Schallreuter et al 2003). However, phosphorylation and acetylation of p53 in vitiligo have not been documented so far. Since both phosphorylation / acetylation are important steps in activation of p53 we tested ATM protein expression in vitiligo *in vitro* melanocytes and in suction blister from patients with vitiligo and controls. The result revealed a stronger expression of ATM under *in vitro* conditions in vitiliginous melanocytes compared to control cells **(Figure 42d and a)**. Overlay with DAPI indicate nuclear localization of ATM which seems to be stronger in vitiligo cells **(Figure 42f and c).**

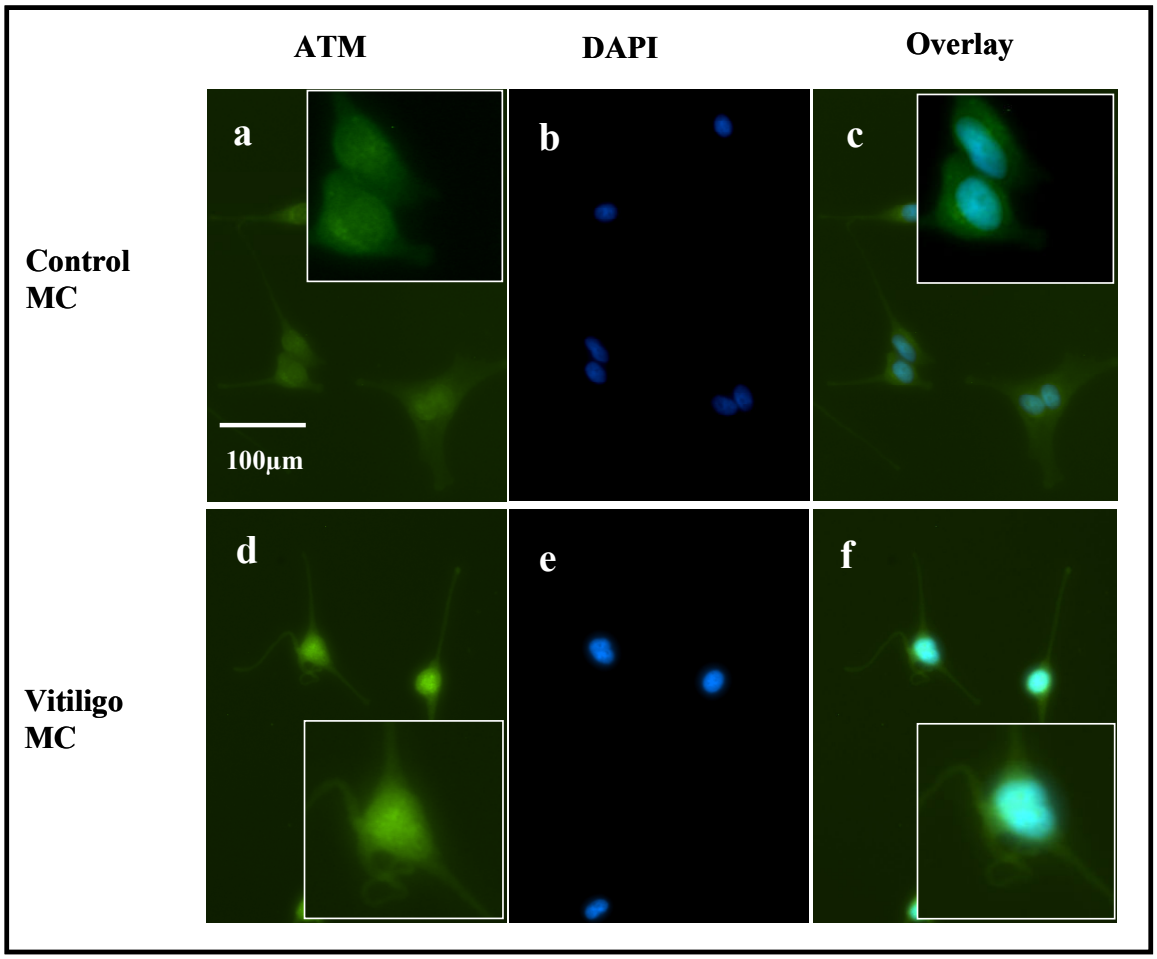


Figure 42
Enhanced ATM expression in vitiliginous melanocytes
Increased ATM expression in vitiliginous melanocytes (d) compared to control cells (a). (b) and (e) DAPI. ATM is also present in the nucleus (f). The expression seemed stronger in this organelle compared to cytosol (c). Magnification x 400, scale bar 100µm

4.8.1.3 Phosphorylation of ser 9 and ser 15 in p53 protein

Next we followed phosphorylation of vitiliginous p53 since it was shown (Canman, Lim et al. 1998; Tibbetts, Brumbaugh et al. 1999) that phosphorylation of ser 9 and ser 15 are due to activity of ATM (Canman, Lim et al. 1998; Tibbetts, Brumbaugh et al. 1999). Western blot analysis of epidermal cell extracts from patients with vitiligo (n=6) and healthy controls (n=2) were revealed phosphorylated ser 9 and ser 15.

Based on these results we can conclude that up-regulated epidermal ATM is functional yielding activation / stabilization of p53 via phosphorylation of ser 9 and ser 15 of the protein.

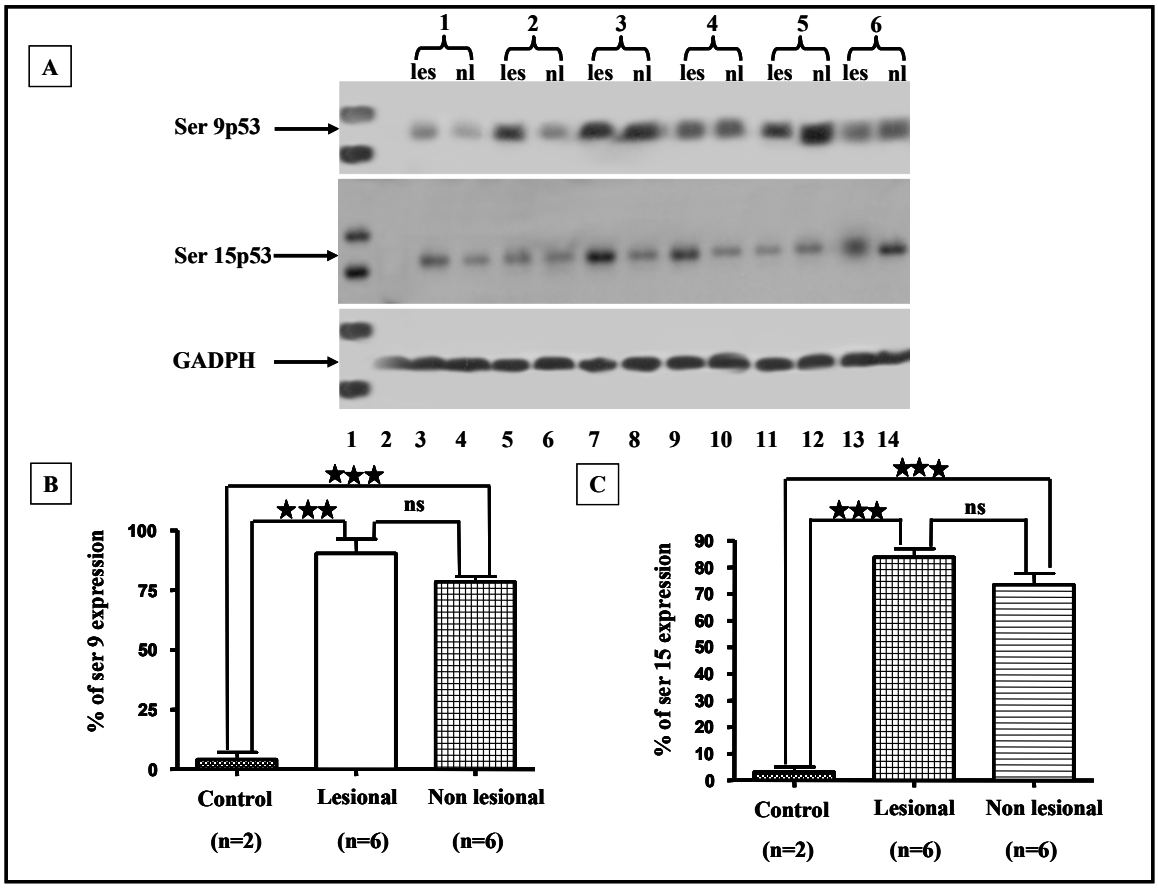


Figure 43

Presence of phosphorylated ser 9 and ser 15 in p53 protein in vitiligo cell extracts

(A) Western blot analysis shows phosphorylation of ser 9 and ser 15 of p53 protein in 6 patients with vitiligo (lanes 3-14). Lane 2 represents cell extract from healthy control. N.B. there is no band detectable. Lane 1 a protein ladder. GADPH is a loading control.

(B and C) Densitometry analysis for phosphorylated ser 9 and ser 15 respectively in correlation to GADPH. Results show the significantly increase of the phosphorylated ser 9 and ser 15 in vitiligo compared to control. (Plots are mean ±SD). (*** $p<0.001$).

4.8.2 Evidence for acetylation of p53 protein in vitiligo

Acetylation is one of the posttranslational modifications for many proteins. PCAF, a histone acetyltransferase (HAT), acetylates lys-370, lys-371, ys-372, lys-373 lys-381, lys-382 of p53 at its C-terminal leading to activation of p53 DNA binding activity (Kouzarides 2000; Sterner and Berger 2000). Acetylation of p53 is also a result of exposing the cell to cellular stress, including DNA damage (Sakaguchi, Watanabe et al. 1998); (Liu, Jin et al. 1999; Pearson, Carbone et al. 2000; Gottifredi and Prives 2001; Ito, Shinkai et al. 2001).

4.8.2.1 Acetylation of lys 373 and lys 382 in p53 protein by PCAF

Consequently we decided to follow this step in more detail by investigating PCAF expression in vitiligo. To do so Western blot analysis was utilised for epidermal suction blister cell extracts from patients with vitiligo (n=6) lesional and non lesional skin and healthy controls (n=2). The results revealed a significant higher expression of PCAF protein in patients (lane 3-14) compared to control (lane 2) **(Figure 44)**. This results suggested that acetylation of p53 could be intact. Western blot analysis was employed to follow selected modification sites on p53 induced by PCAF. The results prove that acetylation of lys 373 and lys 382 in the sequence of p53 protein in vitiligo cell extracts is indeed present (lanes 3-14) meanwhile this residues are absent in control skin. These data imply that acetylation in vitiliginous p53 is not hampered (lane 2). Our earlier data together with these novel data prove the presence of an up-regulated functional / activated p53 in vitiligo (Schallreuter et al 2003).

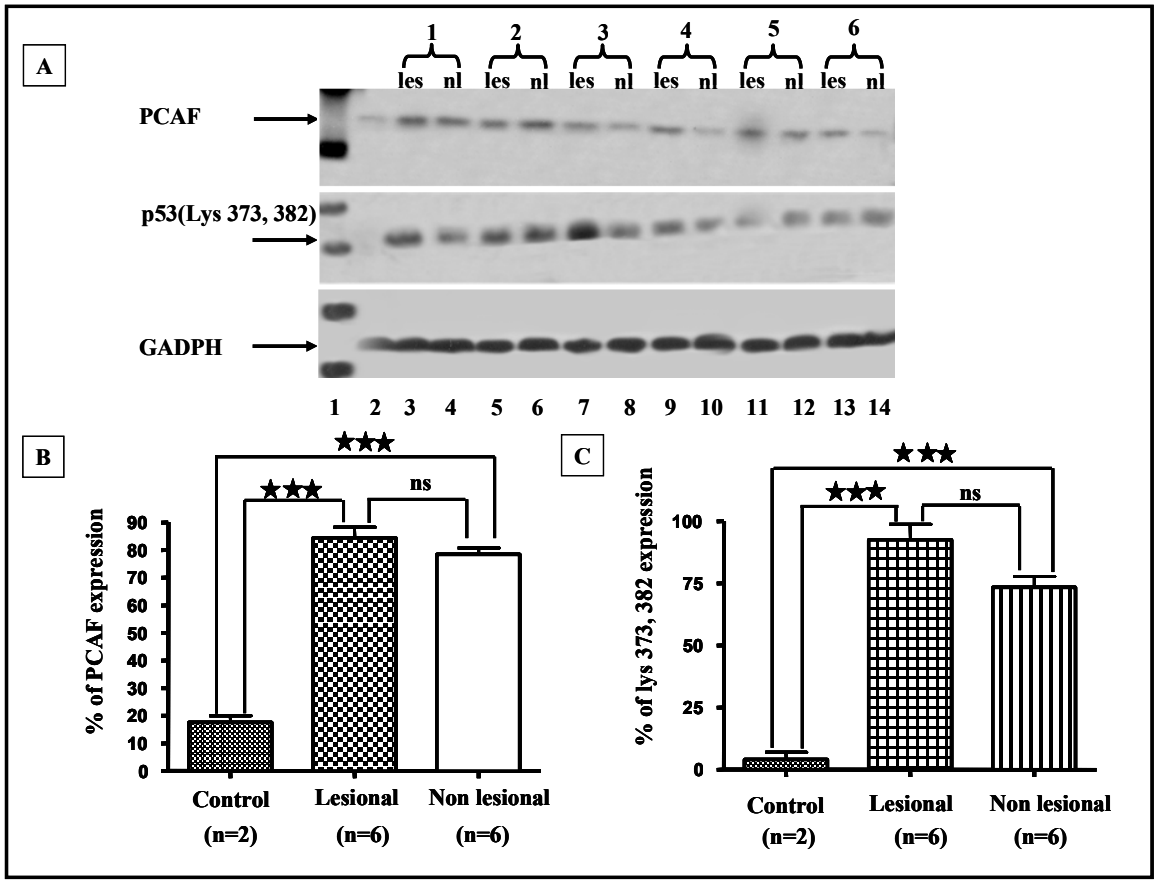


Figure 44
Evidence for acetylation of p53 protein by PCAF in vitiligo
(A) Western blot analysis reveals the over expression of PCAF in vitiligo (lanes 3-14) when compared to control (lane 2). 6 patients have been used in this experiment. Moreover, PCAF protein mediates acetylatoin of lys 373 and lys 382 in the endogenous p53 protein in vitiligo (lanes 3-14) when compared to control (lane 2) While lane 1 represents a protein ladder. GADPH is a loading control.
(B and C) Densitometry analysis of the PCAF protein and acetylated lysine residues respectively in correlation to GADPH. The results demonstrate a significantly higher expression of the PCAF and acetylated lysine residues in vitiligo compared to control. (Plots are mean ±SD). (*** $p<0.001$).

Within this thesis it is demonstrated, for the first time, that up-regulated p53 in vitiligo corresponds with increased phosphorylation in association with increased ATM expression and with increased acetylation in association with increased PCAF expression. These results support our earlier finding on wild type functioning p53 in vitiligo (Schallreuter et al 2003). Moreover, we have shown that the DNA-binding capacity of p53 in vitiligo is enhanced due to contribution of nitration / H_2O_2 mediated oxidation. In addition, we have shown the presence of increased epidermal $p76^{MDM2}$ levels, which could provide an explanation for the constant up-regulation of epidermal p53 in this disease.

4.9 More evidence for functioning of p53 in vitiligo

After showing active p53 in vitiligo we addressed protein functionality. Considering that p53 is a multifunctional protein mediating transcription for proteins involved in cell cycle arrest as well as DNA repair and apoptosis (Lane 1992; Harris 1993; Prives 1994) we chose to follow p21 and PCNA expression under those conditions as found in vitiligo.

4.9.1 Cell cycle arrest is mediated by p53 protein

After activation of p53 the protein mediates induction of p21 which in turn can initiate cell cycle arrest at the G1 phase (Kuerbitz, Plunkett et al. 1992; el-Deiry, Tokino et al. 1993) and G2 phase (Lin and Lowe 2001) in order to stop the replication of the damaged DNA (Hartwell 1992; Hartwell and Kastan 1994). It was reported that if p53 failed to recover DNA damage during cell cycle arrest, it still has the capacity to mediate apoptosis and inhibit replication of the damaged DNA (Hermeking and Eick 1994; Lin and Lowe 2001); (Yonish-Rouach, Resnitzky et al. 1991).

4.9.1.1 Up-regulation of p21 in epidermal cell extracts further support functioning p53 in vitiligo

Expression of p21 in the epidermis of patients with vitiligo was confirmed by Western blotting in epidermal suction blister cell extracts from patients lesional and non lesional skin (n=5) and controls (n=3). The results prove the presence of up-regulated p21 expression **(Figure 45)**. Taken together, this result provides further

support for a functional up-regulated p53 in vitiligo. Notably, evaluation of the bands indicates variable p21 levels between patients **(Figure 45b)**.

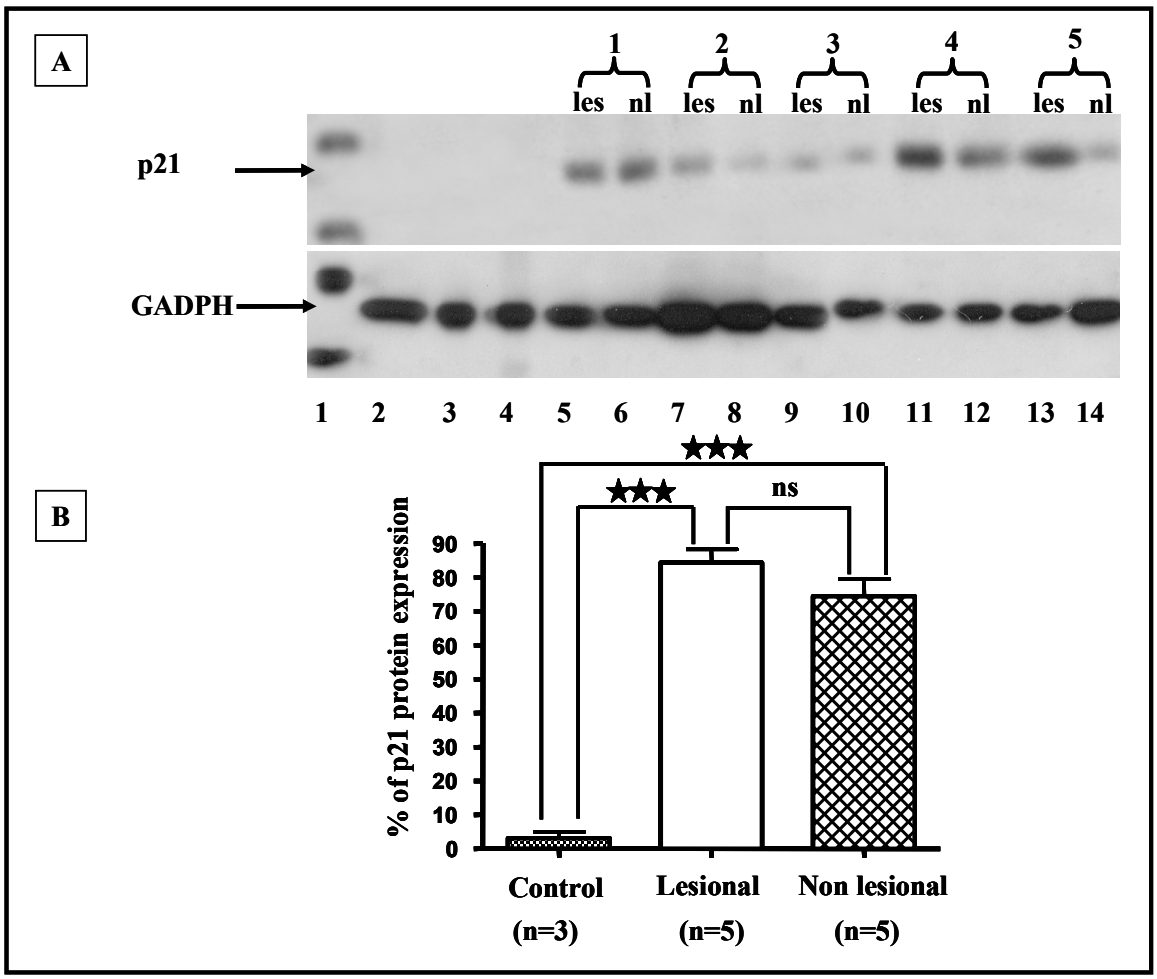


Figure 45
Increased induction of p21 in patients with vitiligo
(A) Western blot demonstrates p21 levels which are significantly different between patients with vitiligo(5) (lanes 5-14) and controls (3) (lanes 2-4). Lane 1, protein ladder. This Western blot was performed along with GADPH, which served as loading control.
(B) Densitometry analysis of the bands in correlation to GADPH proves the presence of high p21 levels in vitiligo which are not detectable in control skin (plots are mean ± SD). (*** $p<0.001$).

4.9.1.2 Increased i*n vitro* expression of p21 protein in epidermal vitiliginous melanocytes

Even under *in vitro* conditions we observed enhanced p21 protein expression compared to control cells **(Figure 46)**. Moreover, p21 was present in the nucleus of vitiliginuos cells **(Figure 46f)**.

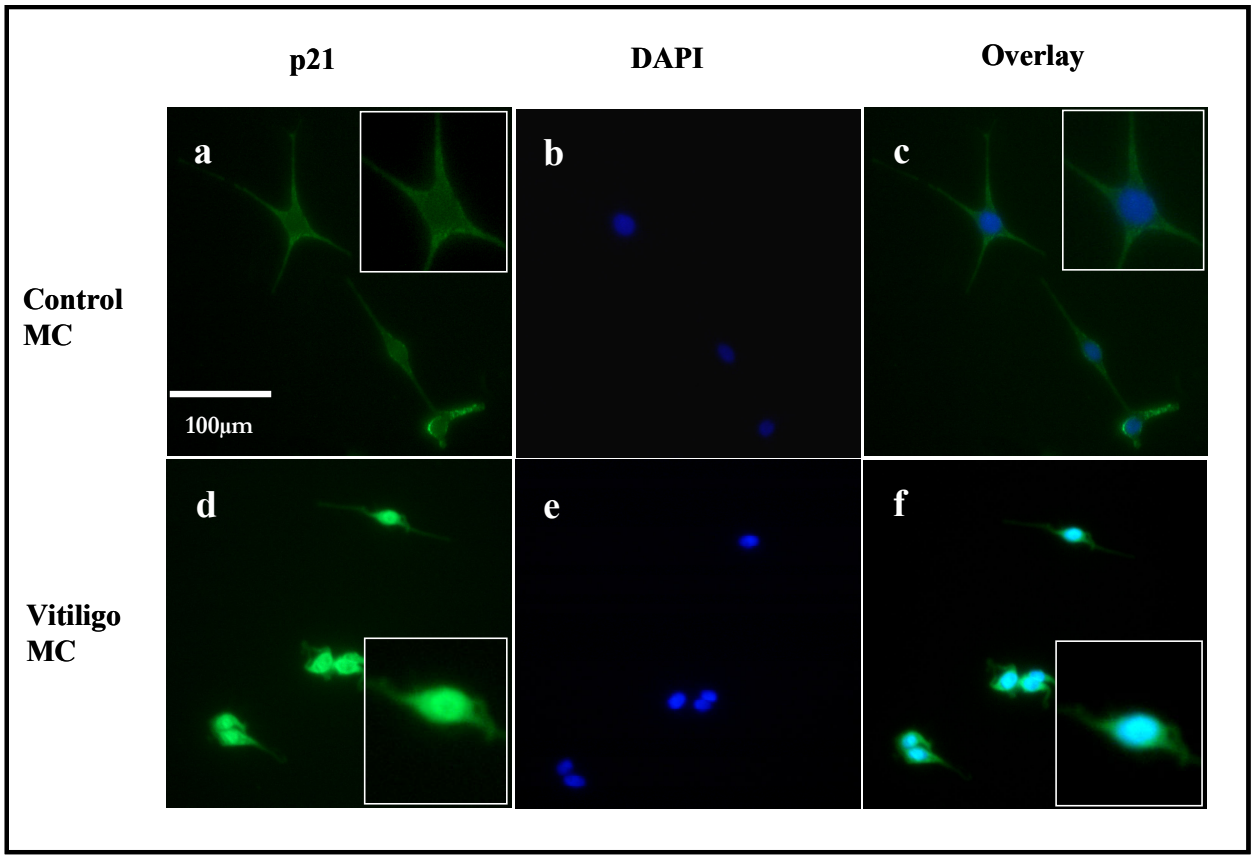


Figure 46
Pronounced p21 expression in vitiligo melanocytes
p21 protein is over expressed in vitiligo melanocytes (d) compared to healthy control melanocytes (a). Overlay of images for control and vitiligo melanocytes with DAPI reveal a high nuclear expression for p21 in vitiligo melanocytes (f) which is absent in control cells (c). Magnification x 400, scale bar 100 μm.

4.9.2.1 Induced PCNA expression in epidermal suction blister cell extracts from patients with vitiligo

Next we examined PCNA expression in the presence of up-regulated p53 in our patients.

Again we used Western blot. The results revealed strongly induced over expression of PCNA in all patients lesional and non lesional skin (n=5) whereas PCNA expression was not detectable in healthy control skin **(Figure 47)**.

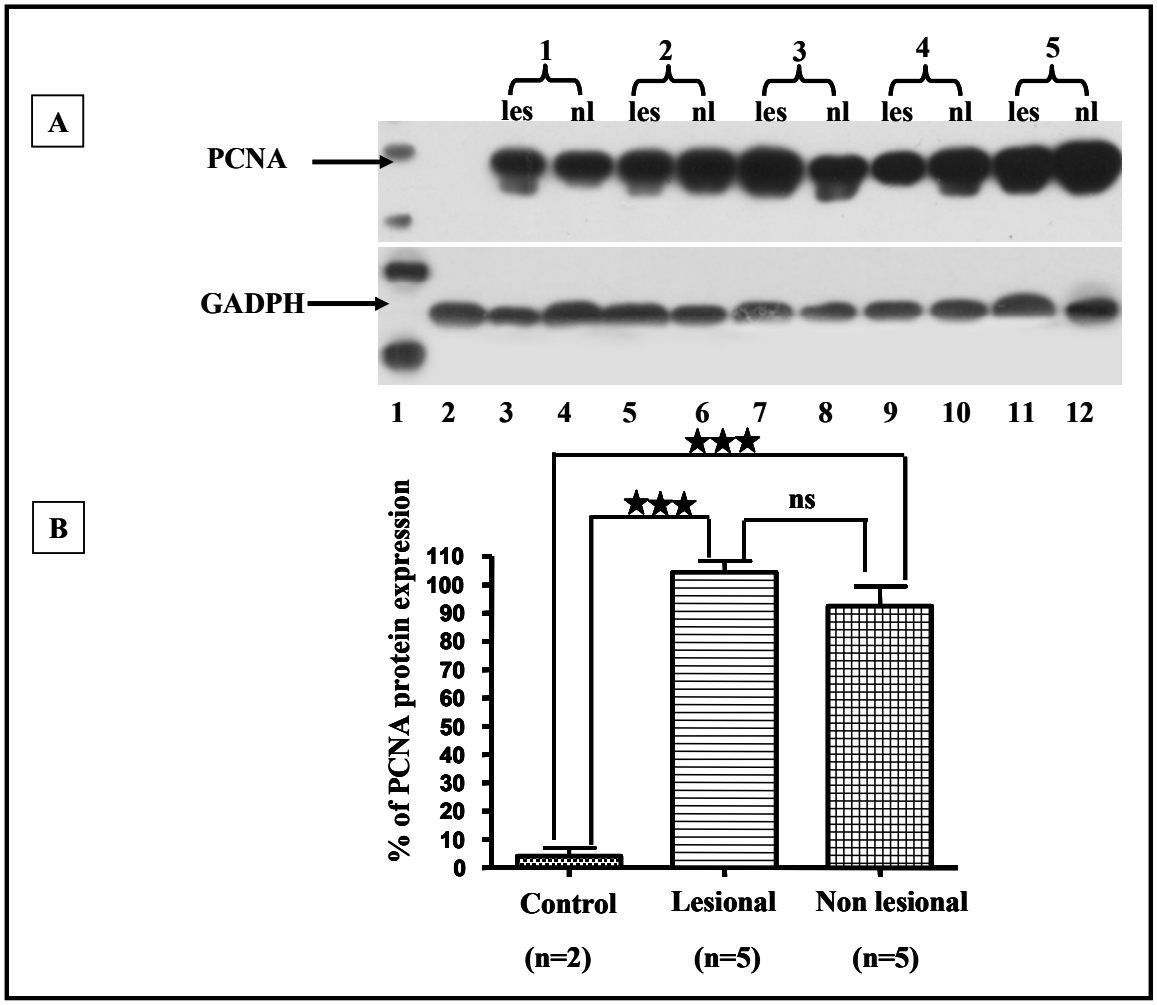


Figure 47
Induction of PCNA in patients with vitiligo.
(A) Western blot analysis reveal high PCNA levels in vitiligo cell extracts (lanes 3-12) which is absent in control skin (lane 2). Lane 1 protein ladder. GADPH was identified to serve as a loading control. Numbers on top of the Western reflect how many patients were used in this investigation.
(B) Densitometry analysis of PCNA bands in correlation to GADPH. PCNA is significantly induced in vitiligo (*** $p<0.001$) (Plots are mean ± SD).

These results indicate that up-regulated PCNA could serve as an effective DNA clamp to promote DNA replication together with DNA polymerase δ controlling, in turn, DNA replication via long patch BER. However, PCNA can also interact directly with APE1 mediating short patch BER (Dianova et al. 2001). P21 is a cycline dependent kinase inhibitor 1, disrupting / blocking DNA replication by binding to PCNA.

4.9.2.2 *In vitro* up-regulation of PCNA protein in epidermal vitiliginous melanocytes

Immuno fluorescence labelling of PCNA indicates that even under *in vitro* conditions this protein is induced in vitiliginous melanocytes, while it is barely detectable in control cells **(Figure 48d)**. PCNA is also highly expressed in the nucleus **(Figure 48f)** compared to control melanocytes **(Figure 48c)**.

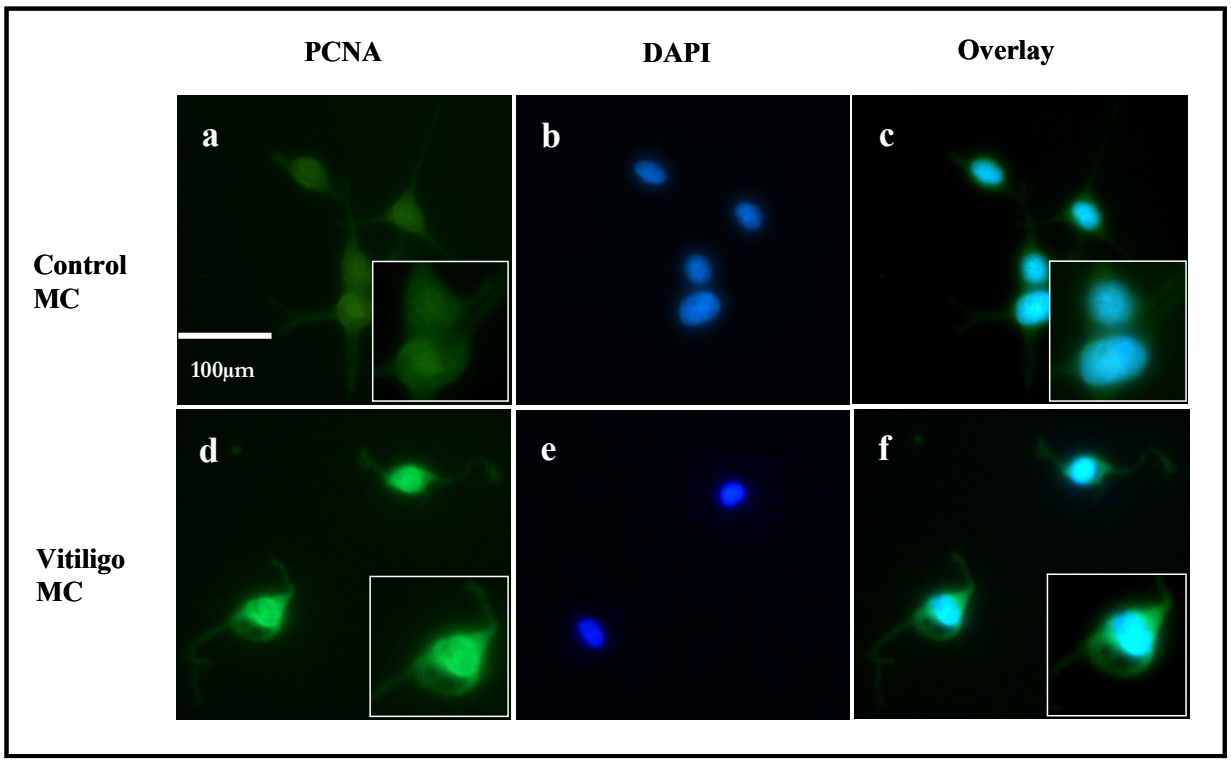


Figure 48
***In vitro* over expression of PCNA in vitiligo melanocytes**
Immuno fluorescence shows increased levels for PCNA protein in vitiligo melanocytes (d) compared to control melanocytes (a). The overlay with DAPI shows nuclear localization for PCNA in vitiligo melanocytes (f). Magnification x 400, scale bar 100 μm.

4.9.3 No place for increased apoptosis in vitiligo

As said already, earlier results on apoptosis in vitiligo are conflicting (Boissy and Nordlund 1997; Kemp, Waterman et al. 2001; Huang, Nordlund et al. 2002; Ruiz-Arguelles, Brito et al. 2007; Schallreuter, Gibbons et al. 2007; Song, Xu et al. 2008) The results of this thesis together with the data of Shalbaf point to the presence of DNA damage in vitiligo as evidenced by increased 8-oxoG levels in the epidermis and in the plasma of these patients (Shalbaf 2009). Our data within the thesis favour a leading role for p53 in the scenario of DNA repair in this disease. However, it was decided to use the same sample material for analysis of BCL-2 because this protein has the ability to inhibit several proapoptotic factors.

Our results identified by Western blot analysis significantly higher expression of BCL-2 in cell extracts of vitiligo (n=5) (lanes 5-14) compared to controls (n=2) (lanes 2 and 3) (*** $p<0.001$). **(Figure 49)**

Together with earlier reports and with the recent data from Shalbaf, who showed absence of caspase 3 and cytochrome c, it is tempting to conclude that in the epidermis of patients with vitiligo is no evidence for increased apoptosis.

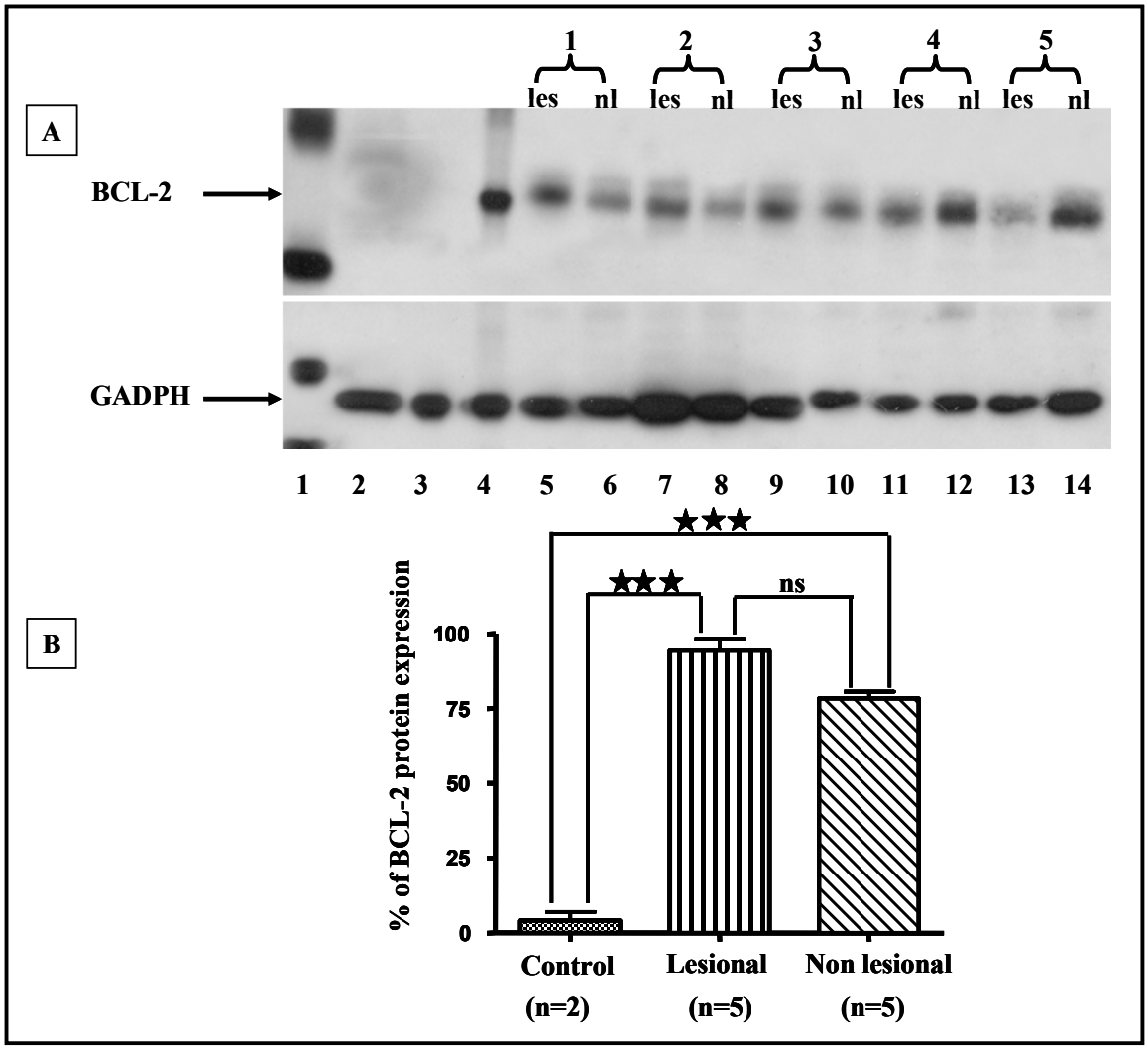


Figure 49
Over expression of epidermal BCL-2 in vitiligo
(A) Western blot shows over expression of BCL-2 in 5 samples of cell extracts of patient's lesional and no lesional skin (n=5) (lanes 5-14) compared to controls (n=2) (lanes 2 and 3) Lane 4 positive control, lane 1 protein ladder. GADPH served as a loading control.
(B) Densitometry analysis in correlation to corresponding GADPH. Data are shown as mean ± SD. BCL-2 expression is significantly higher in patients with vitiligo (*** $p<0.001$).

5.0 DISCUSSION

To date there is convincing evidence that the entire epidermis of patients with vitiligo exhibits oxidative stress based on mM (10^{-3}M) concentrations of H_2O_2 (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008). The first clue in this puzzle originated from low epidermal catalase levels in these patients (Schallreuter, Wood et al. 1991). Over the last decade much work has been concentrating to elucidate the effect of H_2O_2-mediated oxidation in more detail (Schallreuter, Bahadoran et al. 2008). Nowadays there is accumulating evidence that this H_2O_2 mediated stress affects many proteins and peptides in their function due to oxidation of target amino acid residues in their structure including L-methionine, L-tryptophan, L-cysteine and seleno cysteine (Rokos, Beazley et al. 2002; Gillbro, Marles et al. 2004; Hasse, Kothari et al. 2005; Schallreuter, Chavan et al. 2005; Spencer, Chavan et al. 2005; Chavan, Gillbro et al. 2006; Elwary, Chavan et al. 2006; Gibbons, Wood et al. 2006; Schallreuter, Bahadoran et al. 2008; Shalbaf, Gibbons et al. 2008; Wood, Decker et al. 2009). Importantly, epidermal H_2O_2 is transferred to the system, where it affects several enzymes including DHPR (Hasse, Gibbons et al. 2004). Moreover, low catalase levels have been shown in the epidermis as well as in blood cells (Maresca, Flori et al. 2006; Schallreuter, Bahadoran et al. 2008). Recent data from Shalbaf et al revealed that patients with vitiligo produce allantoin which is an excellent marker for H_2O_2-mediated oxidative stress (Shalbaf, Gibbons et al. 2008). In this context it is noteworthy that xanthin oxidase, the enzyme important in this pathway, is activated by low H_2O_2-concentrations, while higher concentrations partially inhibit the enzyme producing in turn allantoin from uric acid (Shalbaf, Gibbons et al. 2008).

Signs for oxidative stress were documented in epidermal cells even under *in vitro* conditions (Medrano and Nordlund 1990; Tobin, Swanson et al. 2000). However, much earlier work by others documented cellular vacuolation in epidermal cells and the deposition of cellular debris (Moellmann, Klein-Angerer et al. 1982; Bhawan and Bhutani 1983). These findings were finally re-interrelated when it was shown that upon reduction of epidermal H_2O_2 by a pseudocatalase PC-KUS this vacuolation disappeared (Tobin, Swanson et al. 2000). One major finding deserved more research. These patients exhibit constant up regulated wild type functioning p53 protein in their skin, while apoptosis could not be detected (Tobin, Swanson et al. 2000; van den Wijngaard, Aten et al. 2000; Schallreuter, Behrens-Williams et al. 2003). Importantly, p53 up regulation is not affected by H_2O_2-reduction as shown in this thesis **(Figure 16).** Taking into consideration that these patients have massive oxidative stress in association with depigmented, but otherwise unremarkable skin, several crucial questions are arising. In this context it is noteworthy that Shalbaf showed for the first time that the skin and the plasma have increased levels of 8-oxo guanine (8-oxoG) which in turn are an excellent footprint for DNA damage (Shalbaf 2009). In this context it is noteworthy that all DNA bases are particularly susceptible to ROS mediated oxidation (Neeley and Essigmann 2006) but the low redox potential of guanine makes this base especially vulnerable leading to several guanine oxidation products. 8-oxoG is the most investigated species and it is often utilised as biomarker for oxidative stress. Failure to remove 8-oxoG before replication leads to G-T transversion mutations (Neeley and Essigmann 2006). Hence, repair mechanisms (GO repair pathway via OGG1) need to be in place.

In this context it is noteworthy that patients with vitiligo have no increased risk for solar induced skin cancer or increased photo damage (Calanchini-Postizzi and Frenk 1987; Westerhof and Schallreuter 1997; Schallreuter, Tobin et al. 2002).

Consequently many questions came into our mind.

- How do the patients combat and repair their DNA damage?
- What could be the role of up regulated p53 in this scenario in vitiligo?
- Why is p53 not degraded by $p90^{MDM2}$?
- What about phosphorylation and acetylation of p53?
- What about down stream signalling via p21 and PCNA? What about apoptosis in vitiligo?
- Is the presence of massive amounts of H_2O_2 and the presence of affecting p53?

The tumour suppressor p53 is considered to be a major effector of the genotoxic stress-signalling pathways which are in part induced by the kinase ATM (Caspari 2000). Here it is noteworthy that ROS mediated DNA damage is related to phosphorylation of p53 by ATM (Akman, O'Connor et al. 2000).

Moreover, it was shown that NO affects both cellular p53 levels and activity. P53 protein is up regulated after exposure to NO leading in turn to DNA damage (Messmer, Ankarcrona et al. 1994; Forrester, Ambs et al. 1996; Messmer and Brune 1996; Calmels, Hainaut et al. 1997). As discussed earlier the deleterious effects of NO are related to the generation of $ONOO^-$ which is an extremely reactive species. The reaction of $ONOO^-$ with proteins leads to the formation of meta nitrotyrosine which in turn can be used as a biomarker for $ONOO^-$ activity (Beckman and Koppenol 1996; Crow and Ischiropoulos 1996).

Given that H_2O_2–stress is present in the skin of patients with vitiligo, we can now add for the first time that these patients have also high levels of $ONOO^-$ in their epidermis as evidenced by the presence of nitro tyrosine in this thesis **(Figure 27).** Consequently we were interested, whether p53 would be affected by H_2O_2 and / or $ONOO^-$. To explore this avenue in more detail, immunofluorescence staining techniques, Western blotting, dot blot analysis, immuno precipitation and electromobility shift assay (EMSA) were utilised. Purification of wild type p53 allowed us to follow kinetics on tyrosine residues in detail.

To confirm up regulated epidermal p53 vitiligo with more improved methodology, we first re-investigated the expression of p53 in this disease (van den Wijngaard, Aten et al. 2000; Schallreuter, Behrens-Williams et al. 2003). In order to follow the effect of H_2O_2 on the protein, we utilised the corresponding catalase expression in the same specimen because expression levels of catalase are excellent biomarkers for H_2O_2 concentrations (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008).

The results confirmed indeed the original data by *in situ* immuno fluorescence and in epidermal suction blister cell extracts from the patients by Western blot (**Figure 16 and 17**) (Schallreuter, Behrens-Williams et al. 2003). However, in this thesis we show both the presence of p53 in the cytosol and in the nucleus. To our surprise we also found up regulated p53 in melanocytes established from patients with vitiligo compared to control cells (**Figure 19**). This discovery unfolds that even under *in vitro* conditions the cells retain this phenomenon. Based on these data it was tempting to invoke stabilisation of p53. This assumption is in agreement with published data that H_2O_2 and other ROS cause stabilization of p53 protein (Akman, O'Connor et al. 2000; Xie, Wang et al. 2001). Validity of our data is supported by the

fact that the antibody binding site on p53 is not affected by H_2O_2 directly as shown by via dot blot analysis **(Figure 18)**. We can conclude that up regulated epidermal p53 is a fact in vitiligo.

Our next question of interest was, why is p53 not degraded in this disease?

5.1 The presence of $p76^{MDM2}$ - the cuase behind up-regulated p53 in vitiligo?

As already mentioned earlier in this thesis, mdm2 acts as an E3 ubiquitin ligase playing an important role in the regulation of p53 by targeting the degradation of this protein (Bueso-Ramos, Yang et al. 1993; Kubbutat, Jones et al. 1997; Fuchs, Adler et al. 1998; Hershko and Ciechanover 1998). Hence, it was a logical step to follow $p90^{MDM2}$ expression in the skin of our patients. Both *in situ* and *in vitro* immuno reactivity revealed the same amount of $p90^{MDM2}$ expression in patients and controls **(Figures 20A and B, Figure 21)**. Hence, the question remained, why was p53 not degraded in vitiligo? Since *mdm2* gene generates many mdm2 protein products, we decided to follow this route, especially since some isoforms bind to the N-terminal domain of p53 protein while others lack this function. The most frequent isoform is $p85/90^{MDM2}$ which contains the entire structure for the main mdm2, $p74/76^{MDM2}$ and $p54/57^{MDM2}$ (Olson, Marechal et al. 1993; Gudas, Nguyen et al. 1995). Importantly $p74/76^{MDM2}$ isoform can not bind to p53 protein because it lacks the N-terminal domain of the full length protein (Olson, Marechal et al. 1993; Haines, Landers et al. 1994). Moreover, $p74/76^{MDM2}$ can antagonise the function of $p85/90^{MDM2}$ to target p53 protein degradation (Perry, Mendrysa et al. 2000). By contrast the $p54/57^{MDM2}$ isoform still has the capacity to bind to p53 protein although the C-terminal domain is lacking (Olson, Marechal et al. 1993). Taken together, the expression level of $p76^{MDM2}$ emerged as a promising candidate to shed some light on the up regulated

p53 in vitiligo. The *in situ* immuno-reactivity results in lesional and non-lesional skin as well as Western blot analysis of epidermal suction blister cell extracts showed indeed high epidermal expression of $p76^{MDM2}$ protein in all patients while healthy controls have hardly any of this isoform (**Figure 22A and B, Figure 23)**. This observation is completely novel. It certainly provides an answer for why p53 protein is not degraded in vitiligo. Our assumption is backed up by the function of $p76^{MDM2}$. It antagonises the binding capacity of $p90^{MDM2}$ to p53 protein (Perry, Mendrysa et al. 2000) while $p76^{MDM2}$ itself can not bind to p53 (Perry, Mendrysa et al. 2000).

To sum up, it is tempting to conclude that $p76^{MDM2}$ inhibits $p90^{MDM2}$ in vitiligo from binding to p53 and therefore p53 is not degraded explaining in turn up regulated p53 in this disease.

5.2 Patients with vitiligo produce NO via iNOS in their epidermis

Earlier it has been documented that NO affects both cellular p53 levels and activity (Messmer, Ankarcrona et al. 1994; Forrester, Ambs et al. 1996; Messmer and Brune 1996; Calmels, Hainaut et al. 1997). Moreover, iNOS is able to produce 10^{-6} M amounts of NO (Nathan and Xie 1994). Since the co-factor for the enzyme (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin is produced in epidermal cells, the generation of NO was an interesting candidate in the scenario of vitiligo (Schallreuter, Wood et al. 1994). *In situ* expression of iNOS demonstrated significantly higher levels throughout the entire epidermis in lesional and non lesional skin compared to control skin **(Figure 24A and B).** This result confirms earlier histochemical data by Nelson (Nelson 1996). Importantly, again iNOS expression is also high under *in vitro* conditions in vitiliginous melanocytes **(Figure 26).** Western blotting of epidermal cell extracts obtained from suction blister roofs confirmed up regulated iNOS in

vitiligo compared to healthy controls **(Figure 25)**. These observations raised the question, whether epidermal NO could lead to formation of $ONOO^-$ in this disease.

5.3 NO generates nitrated tyrosine in vitiligo -a footprint for peroxynitrite ($ONOO^-$)

In this context it has been well established that the $ONOO^-$ is generated by the rapid reaction of NO with $O_2^{\cdot -}$ radical, where the rate of $ONOO^-$ formation depends on the concentration of $O_2^{\cdot -}$ and NO (reviewed in (Radi, Peluffo et al. 2001).

To answer the question of interest, immuno-reactivity for nitrated tyrosine was used as a footprint for $ONOO^-$ production (Beckman and Koppenol 1996; Crow and Ischiropoulos 1996). The results in **Figure 27A and B** revealed significantly higher levels of nitrated tyrosine in all vitiligo samples compared to healthy controls. Moreover, vitiliginous melanocytes expressed high levels of nitrated tyrosine, even under *in vitro* conditions suggesting NO production as a general event in vitiligo **(Figure 28)**.

5.4 Nitration of p53 takes place in vitiligo

Due to the presence of many tyrosine residues in the sequence of p53 it is not surprising that nitration of the protein has been shown by several groups (Messmer, Ankarcrona et al. 1994; Forrester, Ambs et al. 1996; Messmer and Brune 1996; Calmels, Hainaut et al. 1997).

However, to the best of our knowledge, the presence of nitro-tyrosine and nitrated epidermal p53 has never been shown in dermatology so far **(Figure 29A).** The *in situ* results in this thesis prove co-localisation of both p53 and nitro tyrosine in the cytosol as well as in the nucleus throughout the epidermis. The expression was

significantly higher in lesional and non-lesional vitiligo skin compared to controls. These results support that epidermal p53 is nitrated by $ONOO^-$ in vitiligo **(Figure 29B)**. The *in situ* result is further supported in Western blot analysis in epidermal cell extracts of all patients tested **(Figure 30).**

5.5 More evidence for nitration of p53 by $ONOO^-$

In order to get a more detailed understanding on the role of $ONOO^-$ in vitiligo, p53 was produced using the *E. coli* expression system. A T7 RNA polymerase was considered as a re-solublelised active promoter (Midgley, Fisher et al. 1992) allowing production of full length p53 from the *E. coli* strain (BL21/DE3) **(Figure 31, 32, 33 and 34)**. However, some protein degradation was observed, which could be due to the sonication procedure. At the same time there was also production of p53 protein in the non induced cells. This observation has been reported earlier and is most likely due to a leaky *lac* promoter (Studier, Rosenberg et al. 1990). Further experiments demonstrated concentration dependent nitration of p53 **(Figure 35A and B)**. Since $ONOO^-$ is a secondary intermediate anion with a short biological half life, it was important to follow the time course of nitration to judge on the relevance *in vivo* (Thomas, Espey et al. 2002; Bian, Gao et al. 2003). In this thesis we show a time dependent nitration with an optimum at 30 sec using Western blot (**Figure 35C).** These results indicate that $ONOO^-$ is already degraded if the time is >30sec. We also learned that nitration of p53 can occur on relative low levels of $ONOO^-$. Moreover, we obtained an understanding, how long this effect can extend. Most important was the observation that p53 protein was nitrated by $ONOO^-$ and not by NO_2 from decomposed $ONOO^-$.

5.6 Mutant p53 protein in HT-29 cells is also nitrated

The nitration reaction was followed on the mutant p53 protein from HT-29 cells. These cells own a lot of mutant non functional cellular p53 protein **(Figure 36A and B, Figure 37A and B)**. Mutant type p53 was nitrated according to the same protocol as wild type p53. The experiments revealed that the mutant was also nitrated in a dose dependent manner **(Figure 38A and B).** Nitration of p53 has been shown by others (Chazotte-Aubert, Hainaut et al. 2000; Cobbs, Samanta et al. 2001). We can confirm that nitration is a general mechanism in the regulation of p53, regardless whether it is the wild-type or a mutant protein.

In summary, nitration of p53 in epidermal cells is a novel observation. Certainly the high levels of nitrated p53 present in vitiligo skin are adding a new player to the oxidative stress hypothesis in this disease.

5.7 Enhanced DNA binding capacity of p53 in the presence of H_2O_2 and $ONOO^-$ a source for DNA repair in vitiligo?

The presence of H_2O_2 in vitiligo together with ONOO- enphasises a major role of oxidative stress in this disease (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008). In order to get a better understanding of possible implications on the mode of action via p53 under these conditions, we decided to mimic the situation under *in vitro* conditions. Utilising EMSA we learned that $ONOO^-$ abrogated p53-DNA binding capacity at high concentrations >300 μM of this RNS **(Figure 39A)**. We could not confirm that nitration already abrogates p53-DNA binding at concentrations <300μM of $ONOO^-$ (Chazotte-Aubert, Hainaut et al. 2000; Cobbs, Samanta et al. 2001). Surprisingly, oxidation of p53 protein with different concentrations of H_2O_2 (range from 100 μM to 1 mM) revealed that the p53-DNA

binding capacity is not affected by H_2O_2-mediated oxidation **(Figure 39B).** Importantly, we discovered a much higher DNA-binding capacity of p53 after exposure to both $ONOO^-$ and H_2O_2 **(Figure 39C)**.

To our knowledge this observation has not been documented so far. This result also provides additional *in vitro* evidence in support of a functional active p53 in vitiligo as demonstrated earlier in epidermal suction blister cell extracts from patients (Schallreuter, Behrens-Williams et al. 2003).

5.8 Computer simulation supports the *in vitro* finding

To get some more insight into the mechanism as observed by the EMSA results, computer modelling was utilised to follow the effect of nitration and oxidation on the DNA-binding domain of p53. This work was carried out by Dr NCJ Gibbons from our group.

In the native structure of p53 there are seven amino acids (lys 120, ser 241, arg 248, arg 273, ala 276, cys 277 and arg 280) in the DNA binding site which bind 6 nucleotides (Cho, Gorina et al. 1994) **(Figure 40A).** Nitration converts 8 tyrosine to 3-nitrotyrosine in the DNA binding domain of p53 (tyr 103, tyr 106, tyr 126, tyr 163, tyr 206, tyr 220, tyr 234, tyr 236) leading to severe changes in the secondary and tertiary structure. From the 7 residues involved in DNA binding (lys 120, ser 241, arg 248, arg 273, ala276, cys 277 and arg 280) arg 248 and cys 277 are lost yielding a reduced nucleotide binding capacity **(Figure 40B)**. This result is in agreement with our gel shift assay.

After H_2O_2-mediated oxidation the nucleotide binding increases based on the participation of 13 amino acids instead of 7. From the original complex lys 120, arg 248, arg 273, cys 277 and arg 280 are retained, whereas H-bonds from ser 241 and

ala 276 are lost. However, there are now 8 new amino acids (val 122, lys 139, his 179, ser 240, arg 249, pro 250, val 274, arg 283) H-bonding to DNA. This result supports a marked increase in DNA binding **(Figure 40C).** Combined oxidation and nitration of met, trp and tyr residues results in a number of structural changes, but most of the original amino acids remain, including those most crucial to the binding of p53 to DNA. New H-bonding interactions are formed from 10 new amino acids, increasing the number of amino acids interacting to 15. The model predicts the binding of 10 nucleotides in the oxidised complex **(Figure 40D)**.

Taken together, our *in vitro* data from the EMSA experiments is supported by the results from computer simulation.

5.9 Phosphorylation of epidermal p53 is not affected in vitiligo

Phosphorylation is a crucial step for p53 stabilization to perform its function as a transcription factor (Giaccia and Kastan 1998; Appella and Anderson 2001; Ito, Shinkai et al. 2001). In this context it has been shown that phosphorylation of p53 proteins is initiated by DNA damage. Activated ATM/ATR protein kinases are in charge for phosphorylation of 30 amino acids residues at both the amino- and the carboxyl terminus (Canman, Lim et al. 1998; Tibbetts, Brumbaugh et al. 1999; Appella and Anderson 2000). Our results identified up regulated expression of ATM in epidermal cell extracts from the patients **(Figure 41).** Again we found up regulated immuno-reactivity in vitiliginous melanocytes compared to control cells **(Figure 42)**.

Moreover, our investigation proved enzyme activity via phosphorylation of p53 at ser 9 and ser 15 in epidermal cell extracts from all patients with vitiligo by Western blotting **(Figure 43)**.

Based on these results it is temping to conclude that p53 is phosphorylated by ATM in vitiligo leaving a stabilised p53 which is ready to exercise his further functionality on the transcription machinery.

5.10 Epidermal up-regulated p53 is acetylated in patients with vitiligo

Acetylation is one step of posttranslational modifications (Polevoda and Sherman 2000). Therefore we decided to embark on this step by following acetylation of epidermal p53 in vitiligo by choosing histoneacetyl transferase PCAF. Our Western blot results revealed for the first time the presence of this enzyme in epidermal suction blister cell extracts from these patients **(Figure 44)**. The function was proven by the presence of acetylated lysine residues at position 373 and 382 in p53 protein in epidermal cell extracts of all patients compared to control **(Figure 44)**. The presence of acetylated p53 in vitiligo is supported by the work of several groups, who showed that increase in cellular stress leads in turn to increased acetylation of p53 protein (Barlev, Liu et al. 2001; Forsti, Luo et al. 2001; Vaziri, Dessain et al. 2001; Knights, Catania et al. 2006; Kim, Chen et al. 2008; Zhao, Kruse et al. 2008). The up-regulated acetylated p53 is in agreement with work from Luo et al and Brooks et al who demonstrated that PCAF mediates the transcription and activation of p53 protein (Luo, Su et al. 2000; Brooks and Gu 2003).

To sum up, both phosphorylation and acetylation of up regulated p53 are in place in vitiligo highlighting the important role for this protein in the pathogenesis of this disease (Schallreuter, Bahadoran et al. 2008).

5.11 Evidence for p53-mediated cell cycle arrest via p21/PCNA and induction of BER in vitiligo

On the one hand we have an up regulated stabilised (phosphorylated) and acetylated p53 protein in vitiligo, on the other hand we have DNA damage as shown by the presence of significantly increased 8-oxoG levels in the epidermis but even in plasma in all patients tested (Shalbaf 2009).

Therefore it was tempting to have a closer look at some further down stream events. Cell cycle arrest is the first action that p53 performs in a transcription dependent manner to stop replication of DNA damage. It is an important step because it stops the spread of DNA-damage. PCNA is a protein that is essential for DNA replication (Warbrick 2000; Maga and Hubscher 2003; Prosperi 2006), functioning as an aid to DNA polymerase δ allowing efficient attachment and binding to DNA controlling in turn both DNA synthesis and prevention of dissociation from DNA leading to interruption of DNA synthesis. PCNA acts as a DNA clamp. The PCNA trimer assembles on the replication fork of DNA, where it binds DNA polymerase δ and slides along with it to enhance DNA function.

P21, known as cyclin-dependent kinase inhibitor 1, acts to disrupt the replication of DNA by binding to PCNA. The PCNA trimer stays intact and as result of p21 binding does not disassemble. It is thought that p21 blocks key areas on the PCNA that are required to form the DNA replication complex rather than de-stabilising the PCNA trimer. P21 is a 164 amino acid monomer, of which the N-terminus alone confers the ability to bind to PCNA. A 21 amino acid peptide in the C-terminal region has been shown to be able to bind to and inhibit PCNA activity on its own (Pan, Reardon et al. 1995; Gibbs 1997)

Therefore, we selected both p21 and PCNA as major down stream events to get a better understanding of the scenario in vitiligo. We were able to show increased expression of p21 as well as PCNA under *in vitro* conditions by Western blot in epidermal suction blister cell extracts of all patients tested compared to control skin **(Figure 45 and 47)**. To our surprise we were also able to detect increased expression of both p21 and PCNA in vitiliginous melanocytes compared to control cells **(Figure 46 and 48)**. These results imply cell cycle arrest in the G1 and G2 phase in vitiligo because p21 blocks PCNA replication activity. Our assumption is strengthened by up-regulated Gadd45 in the skin of the same patients (el-Deiry, Tokino et al. 1993; Taylor and Stark 2001; Xu, Kim et al. 2002; Shalbaf 2009).

Importantly, both proteins p21 and Gadd45 have the ability to bind to PCNA and stop its DNA replication mechanism enhancing in turn the DNA repair function via base excision repair (BER) (Cahilly-Snyder, Yang-Feng et al. 1987; Flores-Rozas, Kelman et al. 1994; Warbrick 2000; Perucca, Cazzalini et al. 2006). In this context it has been shown by Shalbaf that the DNA glycosylase hOgg1, the enzyme which removes 8-oxoG, is present in vitiligo. However, at present it is unknown, whether this enzyme also removes the corresponding nucleoside 8-OH-dG (Nakano, Kawanishi et al. 2003). Moreover, Shalbaf also showed that DNA polymerase β and APE1 are up-regulated in vitiligo, indicating an efficient BER mechanism in this disease (Shalbaf 2009).

5.12 Absence of increased apoptosis in vitiligo

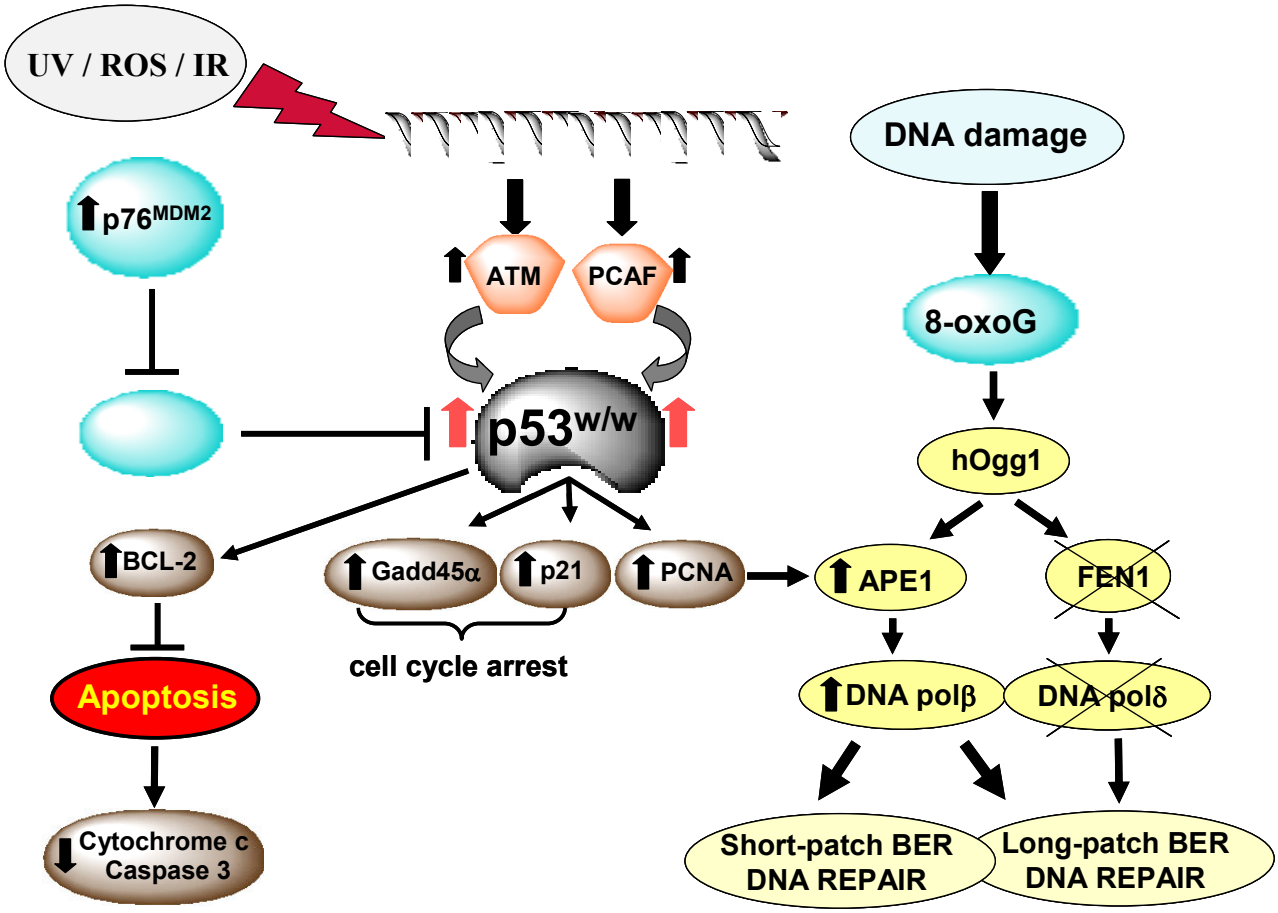
There is an ongoing debate in the scientific community over the presence/absence of increased apoptosis as major mechanism, why melanocytes in vitiligo decrease in numbers and die (Boissy and Nordlund 1997; Kemp, Waterman et al. 2001; Huang,

Nordlund et al. 2002; Ruiz-Arguelles, Brito et al. 2007; Schallreuter, Gibbons et al. 2007; Song, Xu et al. 2008). As mentioned before the results are conflicting. Some groups could not detect any sign for programmed cell death, while others claim to find evidence (Tobin, Swanson et al. 2000; van den Wijngaard, Aten et al. 2000). In their ultra structural studies Tobin and colleagues could neither detect apoptosis on melanocytes and keratinocytes *in situ* in epidermal suction blister sheets nor in melanocytes cell cultures from lesional and non lesional skin despite the presence of vacuolation *in situ* and even under *in vitro* conditions (Tobin, Swanson et al. 2000). Shalbaf re- explored this avenue and he was unable to detect the presence of caspase 3 and cytochrome c in these patients (Shalbaf 2009). This result further supports the absence of the apoptotic pathway. In this thesis we explored also the expression of the anti apoptotic protein BCL-2. The results revealed a highly over expressed protein in epidermal suction blister tissue from all patients tested compared to controls **(Figure 49)**.

Taking into consideration that p90 ^{MDM2} activity is blocked in vitiligo by p76^{MDM2} as shown in this thesis, it seems that apoptosis can not be a major mechanism in vitiligo. Some more support for this concept stems from earlier work of Elwary and colleagues who showed that acetylcholine esterase protein expression and activity is very low in patients with vitiligo compared to healthy controls (Schallreuter, Elwary et al. 2004). Recently this enzyme has been stressed as an apoptosis regulator. Over expression of the enzyme inhibits cell proliferation and promotes apoptosis (Park, Kim et al. 2004; Jiang and Zhang 2008). Since epidermal acetylcholine esterase levels and activities are low in patients with vitiligo, these data also argue against increased apoptosis in vitiligo.

In conclusion, the results of this study favour enhanced DNA repair via short patch BER where p53 is the master regulator in the scenario. Since all findings reported herein were also present on the cellular level in melanocytes from patients, it seems overwhelming evidence that even under *in vitro* conditions a command for efficient DNA repair is in place. Unfortunately we only had one cell line of epidermal melanocytes from a patient with vitiligo. However, since all observations *in situ* and by Western blot were the same in the entire patient group, the data at least suggest that efficient DNA repair is in demand in vitiligo. The strength of the results in this thesis is also based on the number of patients included in the entire analysis. Moreover, all immuno-reactivity studies were carried out in the same patients. All Western blot results are based on the same epidermal suction blister cell extracts. The findings are in all patients the same although the expression varies slightly. The presence of H_2O_2-mediated stress in the mM range was guarded by the intra individual epidermal catalase expression. The data presented herein together with all the data available on oxidative stress in vitiligo provide conclusive evidence for efficient DNA repair machinery in vitiligo. Hence, these results could provide an explanation for the absence of an increased risk for sun induced skin cancer and photo damage in this patient group.

The final view is summarised in **Scheme 1**.



Scheme 1

Up-regulated wild type p53 as the main conductor of ROS-mediated DNA damage/repair in vitiligo

ROS–mediated DNA damage in vitiligo leads to increased 8-oxoG levels in the epidermis and in plasma of patients with vitiligo (Shalbaf 2009). Phosphorylation is a crucial step for p53 stabilization to perform its function as a transcription factor (Giaccia and Kastan 1998; Appella and Anderson 2001; Ito, Shinkai et al. 2001). In this context it has been shown that phosphorylation of p53 protein is initiated by DNA damage. Up-regulated wild type p53 is phosphorylated on ser 9 and ser 15, proving functionality of up-regulated ATM in this disease. Acetylation is one step of posttranslational modifications (Polevoda and Sherman 2000). Up-regulated epidermal PCAF in vitiligo is functioning as shown by the presence of acetylated lysine residues at position 373 and 382, both steps support functioning stabilised p53 as shown earlier (Schallreuter, Behrens-Williams et al. 2003). Under normal conditions p53 is degraded by $p90^{MDM2}$ (Oliner, Kinzler et al. 1992). The isomer $p76^{MDM2}$ binds to $p90^{MDM2}$, preventing binding of $p90^{MDM2}$ to p53, stopping in turn degradation of p53 (Perry, Mendrysa et al. 2000). Our results revealed for the first time high epidermal $p76^{MDM2}$ levels in vitiligo, whereas $p90^{MDM2}$ expression is not affected in this compartment Functioning

stabilised p53 leads to transcription of p21, Gadd45α and PCNA. Both p21 and Gadd45α induce cell cycle arrest and both signals are up regulated in vitiligo (Shalbaf 2009). Oxidative DNA damage is repaired via BER, which is initiated by hOgg1 excising 8-oxoG (Michaels, Tchou et al. 1992; Boiteux 1993; Boiteux and Radicella 1999). Depending on the type of DNA damage, the repair pathways would involve either short or long-patch BER, although these pathways can switch between each other (Wilson and Thompson 1997; Dogliotti, Fortini et al. 2001; Fromme and Verdine 2004). PCNA binds to DNA polymerase δ and works as a processivity factor for this enzyme, which together with FEN1 is involved in long-patch BER (Matsumoto, Kim et al. 1994; Frosina, Fortini et al. 1996; Klungland and Lindahl 1997; Fromme and Verdine 2004). Notably, we were unable to detect epidermal DNA polymerase δ and FEN1 in our patients, but we found up-regulated epidermal APE1 and DNA polymerase β levels (Shalbaf 2009). This result favours short patch BER in vitiligo. However, DNA polymerase β can function in both repair pathways (Dianov, Prasad et al. 1999; Prasad, Dianov et al. 2000). In this context it is of interest that PCNA can interact directly with APE1 (Dianova, Bohr et al. 2001). Moreover, hOgg1, APE1 and DNA polymerase β are directly regulated by p53 (Sengupta and Harris 2005). Therefore, it can be concluded that oxidative DNA damage in vitiligo can be repaired through short-patch BER in cooperation with a specified long-patch BER in the absence of FEN1 and DNA polymerase δ (Shalbaf 2009). Epidermal programmed cell death is ruled out because up-regulated BCL-2 favours anti-apoptotic activity which is further supported by decreased levels of cytochrome c and caspase 3 (Shalbaf 2009). These new findings are further supported by unremarkable levels of the pro-apoptotic stimulus BAX and low acetylcholine esterase levels / activities in vitiligo as shown earlier (van den Wijngaard, Aten et al. 2000; Schallreuter, Elwary et al. 2004). Importantly, the DNA binding capacity of p53 is enhanced by H_2O_2 / $ONOO^-$ oxidation as shown in this thesis. Taken together, patients with vitiligo can efficiently combat epidermal H_2O_2 / $ONOO^-$ induced DNA damage via an up-regulated/activated wild type functioning p53 with a more efficient DNA binding, where p53 is the main conductor in the scenario.

6.0 FUTURE WORK

The results demonstrated in this thesis have introduced a new mechanism in the repair of oxidative DNA damage. Future work in this area should investigate the following points:

- More investigation for the role of PCNA in DNA repair

- Our immunofluorescence results showed the presence of p53 in cytoplasm. More research needs to be carried out to explore the role of p53 in the cytoplasm and nucleus.

- To have a closer look whether there is any correlation between acetylcholine esterase expression and p53 protein in vitiligo

- These novel data could be applied to hair and tumour biology.

- The role of p21 in UV-response needs to be studied in more detail.

- Demonitration of the role of ubiquitin degradation process.

- More clarification for the DNA damage and repair by utilising new assayes.

7.0 REFERENCES

Abdel-Naser, M. B. (1999). "Differential effects on melanocyte growth and melanization of low vs. high calcium keratinocyte-conditioned medium." Br J Dermatol 140(1): 50-5.

Achanta, G. and P. Huang (2004). "Role of p53 in sensing oxidative DNA damage in response to reactive oxygen species-generating agents." Cancer Res 64(17): 6233-9.

Adams, J. D., Jr., L. K. Klaidman, et al. (1997). "Tyrosine hydroxylase: mechanisms of oxygen radical formation." Redox Rep 3(5-6): 273-9.

Adimoolam, S. and J. M. Ford (2002). "p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene." Proc Natl Acad Sci U S A 99(20): 12985-90.

Agoff, S. N., J. Hou, et al. (1993). "Regulation of the human hsp70 promoter by p53." Science 259(5091): 84-7.

Agrawal, D., E. M. Shajil, et al. (2004). "Study on the antioxidant status of vitiligo patients of different age groups in Baroda." Pigment Cell Res 17(3): 289-94.

Akman, S. A., T. R. O'Connor, et al. (2000). "Mapping oxidative DNA damage and mechanisms of repair." Annals of the New York Academy of Sciences 899: 88-102.

Albina, J. E., S. Cui, et al. (1993). "Nitric oxide-mediated apoptosis in murine peritoneal macrophages." J Immunol 150(11): 5080-5.

Appella, E. and C. W. Anderson (2000). "Signaling to p53: breaking the posttranslational modification code." Pathol Biol (Paris) 48(3): 227-45.

Appella, E. and C. W. Anderson (2000). "Signaling to p53: breaking the posttranslational modification code. [Review] [98 refs]." Pathologie Biologie 48(3): 227-45.

Appella, E. and C. W. Anderson (2001). "Post-translational modifications and activation of p53 by genotoxic stresses." Eur J Biochem 268(10): 2764-72.

Arany, I., M. M. Brysk, et al. (1996). "Regulation of inducible nitric oxide synthase mRNA levels by differentiation and cytokines in human keratinocytes." Biochem Biophys Res Commun 220(3): 618-22.

Aronoff, S. (1965). "Catalase: kinetics of photooxidation." Science 150(692): 72-3.

Arrowsmith, C. H. (1999). "Structure and function in the p53 family. [see comments]. [Review] [43 refs]." Cell Death & Differentiation 6(12): 1169-73.

Arrowsmith, C. H. and P. Morin (1996). "New insights into p53 function from structural studies." Oncogene 12(7): 1379-85.

Ashcroft, M. and K. H. Vousden (1999). "Regulation of p53 stability." Oncogene 18(53): 7637-43.

Atoyan, R. Y., A. A. Sharov, et al. (2007). "Oligonucleotide treatment increases eumelanogenesis, hair pigmentation and melanocortin-1 receptor expression in the hair follicle." Exp Dermatol 16(8): 671-7.

Bachinski, L. L., S. E. Olufemi, et al. (2005). "Genetic mapping of a third Li-Fraumeni syndrome predisposition locus to human chromosome 1q23." Cancer Res 65(2): 427-31.

Bae, Y. S., S. W. Kang, et al. (1997). "Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation." J Biol Chem 272(1): 217-21.

Bakalkin, G., T. Yakovleva, et al. (1994). "p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer." Proc Natl Acad Sci U S A 91(1): 413-7.

Bargonetti, J., J. J. Manfredi, et al. (1993). "A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein." Genes Dev 7(12B): 2565-74.

Barlev, N. A., L. Liu, et al. (2001). "Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases." Mol Cell 8(6): 1243-54.

Bartek, J. and J. Lukas (2003). "Chk1 and Chk2 kinases in checkpoint control and cancer." Cancer Cell 3(5): 421-9.

Barzu, O. and M. Dansoreanu (1980). "Spectrophotometric determination of H2O2-generating oxidases using oxyhemoglobin as oxygen donor and indicator." Int J Biochem 11(2): 121-6.

Bates, S. and K. H. Vousden (1996). "p53 in signaling checkpoint arrest or apoptosis." Curr Opin Genet Dev 6(1): 12-8.

Bayle, J. H., B. Elenbaas, et al. (1995). "The carboxyl-terminal domain of the p53 protein regulates sequence- specific DNA binding through its nonspecific nucleic acid-binding activity." Proc Natl Acad Sci U S A 92(12): 5729-33.

Beckman, J. S. and W. H. Koppenol (1996). "Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly." Am J Physiol 271(5 Pt 1): C1424-37.

Bennett, M., K. Macdonald, et al. (1998). "Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis [see comments]." Science 282(5387): 290-3.

Berger, S. L. (2007). "The complex language of chromatin regulation during transcription." Nature 447(7143): 407-12.

Berlett, B. S., B. Friguet, et al. (1996). "Peroxynitrite-mediated nitration of tyrosine residues in Escherichia coli glutamine synthetase mimics adenylylation: relevance to signal transduction." Proc Natl Acad Sci U S A 93(5): 1776-80.

Bhawan, J. and L. K. Bhutani (1983). "Keratinocyte damage in vitiligo." J Cutan Pathol 10(3): 207-12.

Bian, K., Z. Gao, et al. (2003). "The nature of heme/iron-induced protein tyrosine nitration." Proc Natl Acad Sci U S A 100(10): 5712-7.

Blanchard-Fillion, B., J. M. Souza, et al. (2001). "Nitration and inactivation of tyrosine hydroxylase by peroxynitrite." J Biol Chem 276(49): 46017-23.

Blaydes, J. P., V. Gire, et al. (1997). "Tolerance of high levels of wild-type p53 in transformed epithelial cells dependent on auto-regulation by mdm-2." Oncogene 14(15): 1859-68.

Boddy, M. N., P. S. Freemont, et al. (1994). "The p53-associated protein MDM2 contains a newly characterized zinc- binding domain called the RING finger." Trends Biochem Sci 19(5): 198-9.

Boissy, R. E. and J. J. Nordlund (1997). "Molecular basis of congenital hypopigmentary disorders in humans: a review." Pigment Cell Res 10(1-2): 12-24.

Boiteux, S. (1993). "Properties and biological functions of the NTH and FPG proteins of Escherichia coli: two DNA glycosylases that repair oxidative damage in DNA." J Photochem Photobiol B 19(2): 87-96.

Boiteux, S. and J. P. Radicella (1999). "Base excision repair of 8-hydroxyguanine protects DNA from endogenous oxidative stress." Biochimie 81(1-2): 59-67.

Bond, G. L., W. Hu, et al. (2005). "MDM2 is a central node in the p53 pathway: 12 years and counting." Curr Cancer Drug Targets 5(1): 3-8.

Braithwaite, A. W., H. W. Sturzbecher, et al. (1987). "Mouse p53 inhibits SV40 origin-dependent DNA replication." Nature 329(6138): 458-60.

Brooks, C. L. and W. Gu (2003). "Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation." Curr Opin Cell Biol 15(2): 164-71.

Budihardjo, I., H. Oliver, et al. (1999). "Biochemical pathways of caspase activation during apoptosis." Annu Rev Cell Dev Biol 15: 269-90.

Bueso-Ramos, C. E., Y. Yang, et al. (1993). "The human MDM-2 oncogene is overexpressed in leukemias." Blood 82(9): 2617-23.

Burney, S., J. L. Caulfield, et al. (1999). "The chemistry of DNA damage from nitric oxide and peroxynitrite." Mutat Res 424(1-2): 37-49.

Cadwell, C. and G. P. Zambetti (2001). "The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth." Gene 277(1-2): 15-30.

Cahilly-Snyder, L., T. Yang-Feng, et al. (1987). "Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line." Somat. Cell. Mol. Genet. 13: 235-244.

Calanchini-Postizzi, E. and E. Frenk (1987). "Long-term actinic damage in sun-exposed vitiligo and normally pigmented skin." Dermatologica 174(6): 266-71.

Calmels, S., P. Hainaut, et al. (1997). "Nitric oxide induces conformational and functional modifications of wild-type p53 tumor suppressor protein." Cancer Res 57(16): 3365-9.

Cals-Grierson, M. M. and A. D. Ormerod (2004). "Nitric oxide function in the skin." Nitric Oxide 10(4): 179-93.

Canman, C. E., D. S. Lim, et al. (1998). "Activation of the ATM kinase by ionizing radiation and phosphorylation of p53." Science 281(5383): 1677-9.

Casp, C. B., J. X. She, et al. (2002). "Genetic association of the catalase gene (CAT) with vitiligo susceptibility." Pigment Cell Res 15(1): 62-6.

Caspari, T. (2000). "How to activate p53. [Review] " Current Biology 10(8): R315-7.

Cazzalini, O., P. Perucca, et al. (2003). "p21CDKN1A does not interfere with loading of PCNA at DNA replication sites, but inhibits subsequent binding of DNA polymerase delta at the G1/S phase transition." Cell Cycle 2(6): 596-603.

Chavan, B., J. M. Gillbro, et al. (2006). "GTP cyclohydrolase feedback regulatory protein controls cofactor 6-tetrahydrobiopterin synthesis in the cytosol and in the nucleus of epidermal keratinocytes and melanocytes." J Invest Dermatol 126(11): 2481-9.

Chazotte-Aubert, L., P. Hainaut, et al. (2000). "Nitric oxide nitrates tyrosine residues of tumor-suppressor p53 protein in MCF-7 cells." Biochem Biophys Res Commun 267(2): 609-13.

Chehab, N. H., A. Malikzay, et al. (1999). "Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage." Proceedings of the National Academy of Sciences of the United States of America 96(24): 13777-82.

Chen, P. L., Y. M. Chen, et al. (1990). "Genetic mechanisms of tumor suppression by the human p53 gene." Science 250(4987): 1576-80.

Chen, U., S. Chen, et al. (1996). "p21Cip1/Waf1 disrupts the recruitment of human Fen1 by proliferating- cell nuclear antigen into the DNA replication complex." Proc Natl Acad Sci U S A 93(21): 11597-602.

Chipuk, J. E., U. Maurer, et al. (2003). "Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription." Cancer Cell 4(5): 371-81.

Cho, Y., S. Gorina, et al. (1994). "Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations [see comments]." Science 265(5170): 346-55.

Christophers, S. and S. Braver (1987). Elementa Dermatologica.

Cobbs, C. S., J. E. Brenman, et al. (1995). "Expression of nitric oxide synthase in human central nervous system tumors." Cancer Res 55(4): 727-30.

Cobbs, C. S., M. Samanta, et al. (2001). "Evidence for peroxynitrite-mediated modifications to p53 in human gliomas: possible functional consequences." Arch Biochem Biophys 394(2): 167-72.

Cramer, S. F. (1991). "The origin of epidermal melanocytes. Implications for the histogenesis of nevi and melanomas." Arch Pathol Lab Med 115(2): 115-9.

Cross, S. M., C. A. Sanchez, et al. (1995). "A p53-dependent mouse spindle checkpoint." Science 267(5202): 1353-6.

Crow, J. P. and H. Ischiropoulos (1996). "Detection and quantitation of nitrotyrosine residues in proteins: in vivo marker of peroxynitrite." Methods Enzymol 269: 185-94.

Cui, R., H. R. Widlund, et al. (2007). "Central role of p53 in the suntan response and pathologic hyperpigmentation." Cell 128(5): 853-64.

de Rozieres, S., R. Maya, et al. (2000). "The loss of mdm2 induces p53-mediated apoptosis." Oncogene 19(13): 1691-7.

Del Sal, G., E. M. Ruaro, et al. (1995). "Gas1-induced growth suppression requires a transactivation-independent p53 function." Mol Cell Biol 15(12): 7152-60.

Deliconstantinos, G., V. Villiotou, et al. (1996). "Increase of particulate nitric oxide synthase activity and peroxynitrite synthesis in UVB-irradiated keratinocyte membranes." Biochem J 320 (Pt 3): 997-1003.

Deliconstantinos, G., V. Villiotou, et al. (1996). "Nitric oxide and peroxynitrite released by ultraviolet B-irradiated human endothelial cells are possibly involved in skin erythema and inflammation." Exp Physiol 81(6): 1021-33.

Deliconstantinos, G., V. Villiotou, et al. (1995). "Release by ultraviolet B (u.v.B) radiation of nitric oxide (NO) from human keratinocytes: a potential role for nitric oxide in erythema production." Br J Pharmacol 114(6): 1257-65.

Dianov, G. L., R. Prasad, et al. (1999). "Role of DNA polymerase beta in the excision step of long patch mammalian base excision repair." J Biol Chem 274(20): 13741-3.

Dianov, G. L., K. M. Sleeth, et al. (2003). "Repair of abasic sites in DNA." Mutat Res 531(1-2): 157-63.

Dianova, II, V. A. Bohr, et al. (2001). "Interaction of human AP endonuclease 1 with flap endonuclease 1 and proliferating cell nuclear antigen involved in long-patch base excision repair." Biochemistry 40(42): 12639-44.

Dogliotti, E., P. Fortini, et al. (2001). "The mechanism of switching among multiple BER pathways." Prog Nucleic Acid Res Mol Biol 68: 3-27.

Donehower, L. A., M. Harvey, et al. (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature 356(6366): 215-21.

Donehower, L. A., M. Harvey, et al. (1995). "Effects of genetic background on tumorigenesis in p53-deficient mice." Mol Carcinog 14(1): 16-22.

Dornan, D. and T. R. Hupp (2001). "Inhibition of p53-dependent transcription by BOX-I phospho-peptide mimetics that bind to p300." EMBO Reports 2(2): 139-44.

Dumaz, N. and D. W. Meek (1999). "Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2." EMBO Journal 18(24): 7002-10.

Dutta, A., J. M. Ruppert, et al. (1993). "Inhibition of DNA replication factor RPA by p53 [see comments]." Nature 365(6441): 79-82.

el-Deiry, W. S., S. E. Kern, et al. (1992). "Definition of a consensus binding site for p53." Nat Genet 1(1): 45-9.

el-Deiry, W. S., T. Tokino, et al. (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell 75(4): 817-25.

Elias, P. M. (2005). "Stratum corneum defensive functions: an integrated view." J Invest Dermatol 125(2): 183-200.

Elias, P. M. and K. R. Feingold (1992). "Lipids and the epidermal water barrier: metabolism, regulation, and pathophysiology." Semin Dermatol 11(2): 176-82.

Elwary, S. M., B. Chavan, et al. (2006). "The vesicular acetylcholine transporter is present in melanocytes and keratinocytes in the human epidermis." J Invest Dermatol 126(8): 1879-84.

Engel, K., A. Kotlyarov, et al. (1998). "Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation." Embo J 17(12): 3363-71.

Eymin, B., S. Gazzeri, et al. (2002). "Mdm2 overexpression and p14(ARF) inactivation are two mutually exclusive events in primary human lung tumors." Oncogene 21(17): 2750-61.

Fakharzadeh, S. S., S. P. Trusko, et al. (1991). "Tumoriogenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line." EMBO J. 10: 1565-1569.

Feingold, K. R. (2007). "Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis." J Lipid Res 48(12): 2531-46.

Feingold, K. R., M. Q. Man, et al. (1990). "Cholesterol synthesis is required for cutaneous barrier function in mice." J Clin Invest 86(5): 1738-45.

Feng, L., M. Hollstein, et al. (2006). "Ser46 phosphorylation regulates p53-dependent apoptosis and replicative senescence." Cell Cycle 5(23): 2812-9.

Feron, O. (1999). "Endothelial nitric oxide synthase expression and its functionality." Curr Opin Clin Nutr Metab Care 2(4): 291-6.

Fesik, S. W. (2005). "Promoting apoptosis as a strategy for cancer drug discovery." Nat Rev Cancer 5, 876-885.

Finlay, C. A., P. W. Hinds, et al. (1988). "Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life." Mol Cell Biol 8(2): 531-9.

Fitzpatrick, T. B. and A. S. Breathnach (1963). "[the Epidermal Melanin Unit System.]." Dermatol Wochenschr 147: 481-9.

Fitzpatrick, T. B., M. Miyamoto, et al. (1967). "The evolution of concepts of melanin biology." Arch Dermatol 96(3): 305-23.

Flores-Rozas, H., Z. Kelman, et al. (1994). "Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme." Proc Natl Acad Sci U S A 91(18): 8655-9.

Ford, J. M. and P. C. Hanawalt (1997). "Expression of wild-type p53 is required for efficient global genomic nucleotide excision repair in UV-irradiated human fibroblasts." J Biol Chem 272(44): 28073-80.

Forrester, K., S. Ambs, et al. (1996). "Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53." Proc Natl Acad Sci U S A 93(6): 2442-7.

Forsti, A., L. Luo, et al. (2001). "Allelic imbalance on chromosomes 13 and 17 and mutation analysis of BRCA1 and BRCA2 genes in monozygotic twins concordant for breast cancer." Carcinogenesis 22(1): 27-33.

Frebourg, T., N. Barbier, et al. (1995). "Germ-line p53 mutations in 15 families with Li-Fraumeni syndrome." Am J Hum Genet 56(3): 608-15.

Freedman, D. A., L. Wu, et al. (1999). "Functions of the MDM2 oncoprotein. [Review] [79 refs]." Cellular & Molecular Life Sciences 55(1): 96-107.

Friedberg, E. C. (2001). "How nucleotide excision repair protects against cancer." Nat Rev Cancer 1(1): 22-33.

Fromme, J. C. and G. L. Verdine (2004). "Base excision repair." Adv Protein Chem 69: 1-41.

Frosina, G., P. Fortini, et al. (1996). "Two pathways for base excision repair in mammalian cells." J Biol Chem 271(16): 9573-8.

Fuchs, S. Y., V. Adler, et al. (1998). "Mdm2 association with p53 targets its ubiquitination." Oncogene 17(19): 2543-7.

Fukasawa, K., T. Choi, et al. (1996). "Abnormal centrosome amplification in the absence of p53." Science 271(5256): 1744-7.

Fukumura, D., S. Kashiwagi, et al. (2006). "The role of nitric oxide in tumour progression." Nat Rev Cancer 6(7): 521-34.

Funk, W. D., D. T. Pak, et al. (1992). "A transcriptionally active DNA-binding site for human p53 protein complexes." Mol Cell Biol 12(6): 2866-71.

Gavalas, N. G., S. Akhtar, et al. (2006). "Analysis of allelic variants in the catalase gene in patients with the skin depigmenting disorder vitiligo." Biochem Biophys Res Commun 345(4): 1586-91.

Giaccia, A. J. and M. B. Kastan (1998). "The complexity of p53 modulation: emerging patterns from divergent signals." Genes Dev 12(19): 2973-83.

Gibbons, N. C. J., J. M. Wood, et al. (2006). "Computer simulation of native epidermal enzyme structures in the presence and absence of hydrogen peroxide (H_2O_2): Potential and pitfalls." J Invest Dermatol 126(12): 2576-2582.

Gibbs, W. W. (1997). "A cold for cancer." Sci Am 277(2): 36-7.

Gillbro, J. M., L. K. Marles, et al. (2004). "Autocrine catecholamine biosynthesis and the beta-adrenoceptor signal promote pigmentation in human epidermal melanocytes." J Invest Dermatol 123(2): 346-53.

Glickman, M. H. and A. Ciechanover (2002). "The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction." Physiol Rev 82(2): 373-428.

Gobert, C., L. Bracco, et al. (1996). "Modulation of DNA topoisomerase I activity by p53." Biochemistry 35(18): 5778-86.

Goldstein, S. and G. Czapski (1986). "The role and mechanism of metal ions and their complexes in enhancing damage in biological systems or in protecting these systems from the toxicity of O2." J Free Radic Biol Med 2(1): 3-11.

Gottifredi, V. and C. Prives (2001). "Molecular biology. Getting p53 out of the nucleus. [letter; comment]." Science 292(5523): 1851-2.

Grossman, S. R., M. E. Deato, et al. (2003). "Polyubiquitination of p53 by a ubiquitin ligase activity of p300." Science 300(5617): 342-4.

Gu, W. and R. G. Roeder (1997). "Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain." Cell 90(4): 595-606.

Gudas, J., H. Nguyen, et al. (1995). "Effects of cell cycle, wild-type p53 and DNA damage on p21CIP1/Waf1 expression in human breast epithelial cells." Oncogene 11(2): 253-61.

Gudas, J. M., H. Nguyen, et al. (1995). "Differential expression of multiple MDM2 messenger RNAs and proteins in normal and tumorigenic breast epithelial cells." Clin Cancer Res 1(1): 71-80.

Gudkov, A. V. and E. A. Komarova (2003). "The role of p53 in determining sensitivity to radiotherapy." Nat Rev Cancer 3(2): 117-29.

Gulbis, J. M., Z. Kelman, et al. (1996). "Structure of the C-terminal region of p21(WAF1/CIP1) complexed with human PCNA." Cell 87(2): 297-306.

Haavik, J., B. Almas, et al. (1997). "Generation of reactive oxygen species by tyrosine hydroxylase: a possible contribution to the degeneration of dopaminergic neurons?" J Neurochem 68(1): 328-32.

Haber, F. and J. Weiss (1932). "On the catalysis of hydroperxide." Naturwissenschaften 20: 948 - 950.

Hadley, M. E. and W. C. Quevedo, Jr. (1966). "Vertebrate epidermal melanin unit." Nature 209(5030): 1334-5.

Haines, D. S., J. E. Landers, et al. (1994). "Physical and functional interaction between wild-type p53 and mdm2 proteins." Mol Cell Biol 14(2): 1171-8.

Halazonetis, T. D., L. J. Davis, et al. (1993). "Wild-type p53 adopts a 'mutant'-like conformation when bound to DNA." Embo J 12(3): 1021-8.

Halliwell, B. and J. M. Gutteridge (1995). "The definition and measurement of antioxidants in biological systems." Free Radic Biol Med 18(1): 125-6.

Harris, C. C. (1993). "p53: at the crossroads of molecular carcinogenesis and risk assessment." Science 262(5142): 1980-1.

Hartwell, L. (1992). "Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells." Cell 71(4): 543-6.

Hartwell, L. H. and M. B. Kastan (1994). "Cell cycle control and cancer." Science 266(5192): 1821-8.

Harvey, M., M. J. McArthur, et al. (1993). "Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice [see comments]." Nat Genet 5(3): 225-9.

Hasse, S., N. C. Gibbons, et al. (2004). "Perturbed 6-tetrahydrobiopterin recycling via decreased dihydropteridine reductase in vitiligo: more evidence for H2O2 stress." J Invest Dermatol 122(2): 307-13.

Hasse, S., N. C. Gibbons, et al. (2004). "Perturbed 6-tetrahydrobiopterin recycling via decreased dihydropteridine reductase in vitiligo: more evidence for H_2O_2 stress." J Invest Dermatol 122(2): 307-313.

Hasse, S., S. Kothari, et al. (2005). "*In vivo* and *in vitro* evidence for autocrine DCoH/HNF-1alpha transcription of albumin in the human epidermis." Exp Dermatol 14(3): 182-7.

Hattori, Y., C. Nishigori, et al. (1996). "8-hydroxy-2'-deoxyguanosine is increased in epidermal cells of hairless mice after chronic ultraviolet B exposure." J Invest Dermatol 107(5): 733-7.

Haupt, Y., R. Maya, et al. (1997). "Mdm2 promotes the rapid degradation of p53." Nature 387(6630): 296-9.

He, Z., B. T. Brinton, et al. (1993). "The transactivator proteins VP16 and GAL4 bind replication factor A." Cell 73(6): 1223-32.

Henle, F. G. J. (1837). "Symbolae ad anatomiam villorum intestinalum, imprismis eorum epithelii et vasorum lactorum." Berlin.

Hermeking, H. and D. Eick (1994). "Mediation of c-Myc-induced apoptosis by p53." Science 265(5181): 2091-3.

Hershko, A. and A. Ciechanover (1998). "The ubiquitin system." Annu Rev Biochem 67: 425-79.

Hipfner, D. R. and S. M. Cohen (2004). "Connecting proliferation and apoptosis in development and disease." Nat Rev Mol Cell Biol 5(10): 805-15.

Hirao, A., Y. Y. Kong, et al. (2000). "DNA damage-induced activation of p53 by the checkpoint kinase Chk2. [see comments]." Science 287(5459): 1824-7.

Hirobe, T. (2005). "Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes." Pigment Cell Res 18(1): 2-12.

Hoeijmakers, J. H. (2007). "Genome maintenance mechanisms are critical for preventing cancer as well as other aging-associated diseases." Mech Ageing Dev 128(7-8): 460-2.

Hoffman, M. and J. B. Weinberg (1987). "Tumor necrosis factor-alpha induces increased hydrogen peroxide production and Fc receptor expression, but not increased Ia antigen expression by peritoneal macrophages." J Leukoc Biol 42(6): 704-7.

Holbrook, K. A. and K. Wolff (1993). The structure and development of skin. Dermatology in General Medicine. P. F. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg and K. F. Austen, McGraw Hill. 1: 97-145.

Hollstein, M., D. Sidransky, et al. (1991). "p53 mutations in human cancers." Science 253(5015): 49-53.
Huang, C. L., J. J. Nordlund, et al. (2002). "Vitiligo: a manifestation of apoptosis?" Am J Clin Dermatol 3(5): 301-8.
Hupp, T. R., D. W. Meek, et al. (1992). "Regulation of the specific DNA binding function of p53." Cell 71(5): 875-86.
Hwang, B. J., J. M. Ford, et al. (1999). "Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair." Proc Natl Acad Sci U S A 96(2): 424-8.
Ignarro, L. J., G. M. Buga, et al. (1987). "Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide." Proc Natl Acad Sci U S A 84(24): 9265-9.
Imokawa, G., Y. Yada, et al. (1996). "Granulocyte/macrophage colony-stimulating factor is an intrinsic keratinocyte-derived growth factor for human melanocytes in UVA-induced melanosis." Biochem J 313 (Pt 2): 625-31.
Imokawa, G., Y. Yada, et al. (1992). "Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes." J Biol Chem 267(34): 24675-80.
Ito, A., C. H. Lai, et al. (2001). "p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2." Embo J 20(6): 1331-40.
Ito, A., M. Shinkai, et al. (2001). "Radiation-inducible TNF-alpha gene expression under stress-inducible promoter gadd 153 for cancer therapy." J Biosci Bioeng 92(6): 598-601.
Jans, D. A., M. J. Ackermann, et al. (1991). "p34cdc2-mediated phosphorylation at T124 inhibits nuclear import of SV-40 T antigen proteins." J Cell Biol 115(5): 1203-12.
Jenkins, J. R., K. Rudge, et al. (1984). "Cellular immortalization by a cDNA clone encoding the transformation- associated phosphoprotein p53." Nature 312(5995): 651-4.
Jensen, U. B., S. Lowell, et al. (1999). "The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis." Development 126(11): 2409-2418.
Jenuwein, T. and C. D. Allis (2001). "Translating the histone code." Science 293(5532): 1074-80.
Jiang, H. and X. J. Zhang (2008). "Acetylcholinesterase and apoptosis. A novel perspective for an old enzyme." Febs J 275(4): 612-7.
Johnson, A. and M. O'Donnell (2005). "Cellular DNA replicases: components and dynamics at the replication fork." Annu Rev Biochem 74: 283-315.
Kaghad, M., H. Bonnet, et al. (1997). "Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers." Cell 90(4): 809-19.
Kastan, M. B. and J. Bartek (2004). "Cell-cycle checkpoints and cancer." Nature 432(7015): 316-23.
Kastan, M. B. and D. S. Lim (2000). "The many substrates and functions of ATM." Nat Rev Mol Cell Biol 1(3): 179-86.

Kastan, M. B., Q. Zhan, et al. (1992). "A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia." Cell 71(4): 587-97.

Kauser, S., A. J. Thody, et al. (2004). "beta-Endorphin as a regulator of human hair follicle melanocyte biology." J Invest Dermatol 123(1): 184-95.

Kellogg, E. W., 3rd and I. Fridovich (1975). "Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system." J Biol Chem 250(22): 8812-7.

Kemp, C. J., L. A. Donehower, et al. (1993). "Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors." Cell 74(5): 813-22.

Kemp, E. H., E. A. Waterman, et al. (2001). "Autoimmune aspects of vitiligo." Autoimmunity 34(1): 65-77.

Kim, J. E., J. Chen, et al. (2008). "DBC1 is a negative regulator of SIRT1." Nature 451(7178): 583-6.

Klungland, A. and T. Lindahl (1997). "Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1)." Embo J 16(11): 3341-8.

Knights, C. D., J. Catania, et al. (2006). "Distinct p53 acetylation cassettes differentially influence gene-expression patterns and cell fate." J Cell Biol 173(4): 533-44.

Knippschild, U., D. M. Milne, et al. (1997). "p53 is phosphorylated in vitro and in vivo by the delta and epsilon isoforms of casein kinase 1 and enhances the level of casein kinase 1 delta in response to topoisomerase-directed drugs." Oncogene 15(14): 1727-36.

Ko, L. J. and C. Prives (1996). "p53: puzzle and paradigm." Genes Dev 10(9): 1054-72.

Konishi, A., S. Shimizu, et al. (2003). "Involvement of histone H1.2 in apoptosis induced by DNA double-strand breaks." Cell 114(6): 673-88.

Kouzarides, T. (2000). "Acetylation: a regulatory modification to rival phosphorylation?" Embo J 19(6): 1176-9.

Kraiss, S., A. Quaiser, et al. (1988). "Oligomerization of oncoprotein p53." J Virol 62(12): 4737-44.

Kubbutat, M. H., S. N. Jones, et al. (1997). "Regulation of p53 stability by Mdm2." Nature 387(6630): 299-303.

Kuerbitz, S. J., B. S. Plunkett, et al. (1992). "Wild-type p53 is a cell cycle checkpoint determinant following irradiation." Proc Natl Acad Sci U S A 89(16): 7491-5.

Kurzen, H. and K. U. Schallreuter (2004). "Novel aspects in cutaneous biology of acetylcholine synthesis and acetylcholine receptors." Exp Dermatol 13 Suppl 4: 27-30.

Lambert, P. F., F. Kashanchi, et al. (1998). "Phosphorylation of p53 serine 15 increases interaction with CBP." J Biol Chem 273(49): 33048-53.

Lane, D. P. (1992). "Cancer - P53, Guardian of the Genome." 358(6381): 15-16.

Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature 358(6381): 15-6.

Lane, D. P. (1993). "Cancer. A death in the life of p53 [news; comment]." Nature 362(6423): 786-7.

Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." Nature 278(5701): 261-3.

Lavker, R. M. and T. T. Sun (1982). "Heterogeneity in epidermal basal keratinocytes: Morphological and functional correlations." Science 215: 1239-1240.

Le Poole, I. C., R. M. van den Wijngaard, et al. (1993). "Presence or absence of melanocytes in vitiligo lesions: an immunohistochemical investigation." J Invest Dermatol 100(6): 816-22.

Lee, S., B. Elenbaas, et al. (1995). "p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches." Cell 81(7): 1013-20.

Lee, S. D. and E. Alani (2006). "Analysis of interactions between mismatch repair initiation factors and the replication processivity factor PCNA." J Mol Biol 355(2): 175-84.

Leveillard, T., L. Andera, et al. (1996). "Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations." Embo J 15(7): 1615-24.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell 88(3): 323-31.

Levrero, M., V. De Laurenzi, et al. (1999). "Structure, function and regulation of p63 and p73. [see comments]. [Review] [43 refs]." Cell Death & Differentiation 6(12): 1146-53.

Levrero, M., V. De Laurenzi, et al. (2000). "The p53/p63/p73 family of transcription factors: overlapping and distinct functions. [Review] [78 refs]." Journal of Cell Science 113(Pt 10): 1661-70.

Li, A. G., L. G. Piluso, et al. (2007). "An acetylation switch in p53 mediates holo-TFIID recruitment." Mol Cell 28(3): 408-21.

Li, M., C. L. Brooks, et al. (2003). "Mono- versus polyubiquitination: differential control of p53 fate by Mdm2." Science 302(5652): 1972-5.

Li, R. and M. R. Botchan (1993). "The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication." Cell 73(6): 1207-21.

Lieber, M. R., Y. Ma, et al. (2003). "Mechanism and regulation of human non-homologous DNA end-joining." Nat Rev Mol Cell Biol 4(9): 712-20.

Lin, A. W. and S. W. Lowe (2001). "Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation." Proc Natl Acad Sci U S A 98(9): 5025-30.

Lin, B., S. K. Kolluri, et al. (2004). "Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3." Cell 116(4): 527-40.

Lin, J., J. Chen, et al. (1994). "Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein." Genes Dev 8(10): 1235-46.

Liu, Q., C. Jin, et al. (1999). "The Binding Interface between an E2 (UBC9) and a Ubiquitin Homologue (UBL1)." J Biol Chem 274(24): 16979-16987.

Lu, H. and A. J. Levine (1995). "Human TAFII31 protein is a transcriptional coactivator of the p53 protein." Proc Natl Acad Sci U S A 92(11): 5154-8.

Lu, X. and D. P. Lane (1993). "Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes?" Cell 75(4): 765-78.

Luo, J., A. Y. Nikolaev, et al. (2001). "Negative control of p53 by Sir2alpha promotes cell survival under stress." Cell 107(2): 137-48.

Luo, J., F. Su, et al. (2000). "Deacetylation of p53 modulates its effect on cell growth and apoptosis." Nature 408(6810): 377-81.

Maga, G. and U. Hubscher (2003). "Proliferating cell nuclear antigen (PCNA): a dancer with many partners." J Cell Sci 116(Pt 15): 3051-60.

Majka, J. and P. M. Burgers (2004). "The PCNA-RFC families of DNA clamps and clamp loaders." Prog Nucleic Acid Res Mol Biol 78: 227-60.

Maker, H. S., C. Weiss, et al. (1981). "Coupling of dopamine oxidation (monoamine oxidase activity) to glutathione oxidation via the generation of hydrogen peroxide in rat brain homogenates." J Neurochem 36(2): 589-93.

Malkin, D., F. P. Li, et al. (1990). "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms [see comments]." Science 250(4985): 1233-8.

Mannick, J. B. and C. M. Schonhoff (2004). "NO means no and yes: regulation of cell signaling by protein nitrosylation." Free Radic Res 38(1): 1-7.

Marchenko, N. D., A. Zaika, et al. (2000). "Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling." J Biol Chem 275(21): 16202-12.

Marechal, V., B. Elenbaas, et al. (1997). "Conservation of structural domains and biochemical activities of the MDM2 protein from Xenopus laevis." Oncogene 14(12): 1427-33.

Maresca, V., E. Flori, et al. (2006). "UVA-induced modification of catalase charge properties in the epidermis is correlated with the skin phototype." J Invest Dermatol 126(1): 182-90.

Martinoli, C., E. Zocchi, et al. (1984). "Progesterone enhances reactive oxygen intermediates production by cultured human monocytes." Boll Soc Ital Biol Sper 60(10): 1871-7.

Matsumoto, Y., K. Kim, et al. (1994). "Proliferating cell nuclear antigen-dependent abasic site repair in Xenopus laevis oocytes: an alternative pathway of base excision DNA repair." Mol Cell Biol 14(9): 6187-97.

Medrano, E. E. and J. J. Nordlund (1990). "Successful culture of adult human melanocytes obtained from normal and vitiligo donors." J Invest Dermatol 95(4): 441-5.

Meek, D. W. (1999). "Mechanisms of switching on p53: a role for covalent modification?. [Review] [91 refs]." Oncogene 18(53): 7666-75.

Melino, G., F. Bernassola, et al. (2004). "p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation." J Biol Chem 279(9): 8076-83.

Merchant, A. K., T. L. Loney, et al. (1996). "Expression of wild-type p53 stimulates an increase in both Bax and Bcl- xL protein content in HT29 cells." Oncogene 13(12): 2631-7.

Messmer, U. K., M. Ankarcrona, et al. (1994). "p53 expression in nitric oxide-induced apoptosis." FEBS Lett 355(1): 23-6.

Messmer, U. K. and B. Brune (1996). "Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways." Biochem J 319(Pt 1): 299-305.

Michael, D. and M. Oren (2003). "The p53-Mdm2 module and the ubiquitin system." Semin Cancer Biol 13(1): 49-58.

Michaels, M. L., J. Tchou, et al. (1992). "A repair system for 8-oxo-7,8-dihydrodeoxyguanine." Biochemistry 31(45): 10964-8.
Midgley, C. A., C. J. Fisher, et al. (1992). "Analysis of p53 expression in human tumours: an antibody raised against human p53 expressed in Escherichia coli." J Cell Sci 101(Pt 1): 183-9.
Mihara, M., S. Erster, et al. (2003). "p53 has a direct apoptogenic role at the mitochondria." Mol Cell 11(3): 577-90.
Miller, C. W., A. Aslo, et al. (1990). "Frequency and structure of p53 rearrangements in human osteosarcoma." Cancer Res 50(24): 7950-4.
Mills, A. A., B. Zheng, et al. (1999). "p63 is a p53 homologue required for limb and epidermal morphogenesis." Nature 398(6729): 708-13.
Miyashita, T. (1997). "[p53-mediated apoptosis]." Tanpakushitsu Kakusan Koso 42(10 Suppl): 1657-63.
Miyashita, T., M. Harigai, et al. (1994). "Identification of a p53-dependent negative response element in the bcl- 2 gene." Cancer Res 54(12): 3131-5.
Miyashita, T., S. Krajewski, et al. (1994). "Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo." Oncogene 9(6): 1799-805.
Miyashita, T. and J. C. Reed (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." Cell 80(2): 293-9.
Moellmann, G., S. Klein-Angerer, et al. (1982). "Extracellular granular material and degeneration of keratinocytes in the normally pigmented epidermis of patients with vitiligo." J Invest Dermatol 79(5): 321-30.
Momand, J., G. P. Zambetti, et al. (1992). "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation." Cell 69(7): 1237-45.
Moochhala, S. and A. Rajnakova (1999). "Role of nitric oxide in cancer biology." Free Radic Res 31(6): 671-9.
Nagata, S. (1997). "[Apoptosis and diseases]." Nippon Naika Gakkai Zasshi 86(9): 1579-83.
Nakano, M., Y. Kawanishi, et al. (2003). "Oxidative DNA damage (8-hydroxydeoxyguanosine) and body iron status: a study on 2507 healthy people." Free Radic Biol Med 35(7): 826-32.
Nathan, C. and Q. W. Xie (1994). "Nitric oxide synthases: roles, tolls, and controls." Cell 78(6): 915-8.
Neeley, W. L. and J. M. Essigmann (2006). "Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products." Chem Res Toxicol 19(4): 491-505.
Nelson, L. R. (1996). "Nitric oxide synthase in vitiligo." M.Sc thesis, University of Bradford.
Nyberg, K. A., R. J. Michelson, et al. (2002). "Toward maintaining the genome: DNA damage and replication checkpoints." Annu Rev Genet 36: 617-56.
Oberprieler, N. G. (2007). The influence of nitric oxide and peroxynitrite on integrin A and B mediated platelet adhesion / nitric oxide inhibits platelet adhesion to immobilised fibrinogen by regulating both inside-out and outside-in signalling cascades in a cGMP-independent manner. Inside-out signalling events are augmented by peroxynitrite-induced dense granule secretion. Biomedical Science Department, University of Bradford.

Oda, K., H. Arakawa, et al. (2000). "p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53." Cell 102(6): 849-62.

Oliner, J. D., K. W. Kinzler, et al. (1992). "Amplification of a gene encoding a p53-associated protein in human sarcomas [see comments]." Nature 358(6381): 80-3.

Oliner, J. D., J. A. Pietenpol, et al. (1993). "Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53." Nature 362(6423): 857-60.

Olson, D. C., V. Marechal, et al. (1993). "Identification and characterization of multiple mdm-2 proteins and mdm- 2-p53 protein complexes." Oncogene 8(9): 2353-60.

Olson, J. S., D. P. Ballow, et al. (1974). "The reaction of xanthine oxidase with molecular oxygen." J Biol Chem 249(14): 4350-62.

Oren, M. (1985). "The p53 cellular tumor antigen: gene structure, expression and protein properties." Biochim Biophys Acta 823(1): 67-78.

Oren, M. (1994). "Relationship of p53 to the control of apoptotic cell death." Semin Cancer Biol 5(3): 221-7.

Ormerod, A. D., P. Copeland, et al. (1999). "The inflammatory and cytotoxic effects of a nitric oxide releasing cream on normal skin." J Invest Dermatol 113(3): 392-7.

Ortonne, J. P. and S. K. Bose (1993). "Vitiligo: where do we stand?" Pigment Cell Res 6(2): 61-72.

Ozaki, K., T. Sukata, et al. (1998). "High susceptibility of p53(+/-) knockout mice in N-butyl-N-(4- hydroxybutyl)nitrosamine urinary bladder carcinogenesis and lack of frequent mutation in residual allele." Cancer Res 58(17): 3806-11.

Pan, Z. Q., J. T. Reardon, et al. (1995). "Inhibition of nucleotide excision repair by the cyclin-dependent kinase inhibitor p21." J Biol Chem 270(37): 22008-16.

Park, H. H., E. Ha, et al. (2006). "Association study between catalase gene polymorphisms and the susceptibility to vitiligo in Korean population." Exp Dermatol 15(5): 377-80.

Park, S. E., N. D. Kim, et al. (2004). "Acetylcholinesterase plays a pivotal role in apoptosome formation." Cancer Res 64(8): 2652-5.

Parks, D. A. and D. N. Granger (1986). "Xanthine oxidase: biochemistry, distribution and physiology." Acta Physiol Scand Suppl 548: 87-99.

Pavletich, N. P., K. A. Chambers, et al. (1993). "The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots." Genes Dev 7(12B): 2556-64.

Pearson, M., R. Carbone, et al. (2000). "PML regulates p53 acetylation and premature senescence induced by oncogenic Ras." Nature 406(6792): 207-10.

Pelengaris, S. and M. Khan (2006). "The molecular biology of cancer." Blackwell publishing Ltd.

Perry, M. E., S. M. Mendrysa, et al. (2000). "p76(MDM2) inhibits the ability of p90(MDM2) to destabilize p53." J Biol Chem 275(8): 5733-8.

Perry, M. E., S. M. Mendrysa, et al. (2000). "p76(MDM2) inhibits the ability of p90(MDM2) to destabilize p53." Journal of Biological Chemistry 275(8): 5733-8.

Perry, M. E., J. Piette, et al. (1993). "The mdm-2 gene is induced in response to UV light in a p53-dependent manner." Proc Natl Acad Sci U S A 90(24): 11623-7.

Perucca, P., O. Cazzalini, et al. (2006). "Spatiotemporal dynamics of p21CDKN1A protein recruitment to DNA-damage sites and interaction with proliferating cell nuclear antigen." J Cell Sci 119(Pt 8): 1517-27.

Petitjean, A., E. Mathe, et al. (2007). "Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database." Hum Mutat 28(6): 622-9.

Picksley, S. M., B. Vojtesek, et al. (1994). "Immunochemical analysis of the interaction of p53 with MDM2;--fine mapping of the MDM2 binding site on p53 using synthetic peptides." Oncogene 9(9): 2523-9.

Polevoda, B. and F. Sherman (2000). "Nalpha -terminal acetylation of eukaryotic proteins." J Biol Chem 275(47): 36479-82.

Poli, G., G. Leonarduzzi, et al. (2004). "Oxidative stress and cell signalling." Curr Med Chem 11(9): 1163-82.

Prasad, R., G. L. Dianov, et al. (2000). "FEN1 stimulation of DNA polymerase beta mediates an excision step in mammalian long patch base excision repair." J Biol Chem 275(6): 4460-6.

Prives, C. (1994). "How loops, beta sheets, and alpha helices help us to understand p53." Cell 78(4): 543-6.

Prives, C., J. Bargonetti, et al. (1994). "DNA-binding properties of the p53 tumor suppressor protein." Cold Spring Harb Symp Quant Biol 59: 207-13.

Prives, C. and P. A. Hall (1999). "The P53 pathway." Journal of Pathology 187(1): 112-126.

Prokocimer, M. and V. Rotter (1994). "Structure and function of p53 in normal cells and their aberrations in cancer cells: projection on the hematologic cell lineages." Blood 84(8): 2391-411.

Prosperi, E. (2006). "The fellowship of the rings: distinct pools of proliferating cell nuclear antigen trimer at work." Faseb J 20(7): 833-7.

Qureshi, A. A., J. Hosoi, et al. (1996). "Langerhans cells express inducible nitric oxide synthase and produce nitric oxide." J Invest Dermatol 107(6): 815-21.

Radi, R. (2004). "Nitric oxide, oxidants, and protein tyrosine nitration." Proc Natl Acad Sci U S A 101(12): 4003-8.

Radi, R., G. Peluffo, et al. (2001). "Unraveling peroxynitrite formation in biological systems." Free Radic Biol Med 30(5): 463-88.

Rawlingson, A., S. A. Greenacre, et al. (2000). "Generation of peroxynitrite in localised, moderate temperature burns." Burns 26(3): 223-7.

Raycroft, L., H. Y. Wu, et al. (1990). "Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene." Science 249(4972): 1049-51.

Reed, M., B. Woelker, et al. (1995). "The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation." Proc Natl Acad Sci U S A 92(21): 9455-9.

Rihs, H. P. and R. Peters (1989). "Nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of the Simian virus 40 T-antigen." Embo J 8(5): 1479-84.

Riley, T., E. Sontag, et al. (2008). "Transcriptional control of human p53-regulated genes." Nat Rev Mol Cell Biol 9(5): 402-12.

Rocha, I. M. and L. A. Guillo (2001). "Lipopolysaccharide and cytokines induce nitric oxide synthase and produce nitric oxide in cultured normal human melanocytes." Arch Dermatol Res 293(5): 245-8.

Rodenas, J., T. Carbonell, et al. (2000). "Different roles for nitrogen monoxide and peroxynitrite in lipid peroxidation induced by activated neutrophils." Free Radic Biol Med 28(3): 374-80.

Rokos, H., W. D. Beazley, et al. (2002). "Oxidative stress in vitiligo: photo-oxidation of pterins produces H(2)O(2) and pterin-6-carboxylic acid." Biochem Biophys Res Commun 292(4): 805-11.

Rossi, F., V. Della Bianca, et al. (1985). "Mechanisms and functions of the oxygen radicals producing respiration of phagocytes." Comp Immunol Microbiol Infect Dis 8(2): 187-204.

Rovinski, B., D. Munroe, et al. (1987). "Deletion of 5'-coding sequences of the cellular p53 gene in mouse erythroleukemia: a novel mechanism of oncogene regulation." Mol Cell Biol 7(2): 847-53.

Ruiz-Arguelles, A., G. J. Brito, et al. (2007). "Apoptosis of melanocytes in vitiligo results from antibody penetration." J Autoimmun 29(4): 281-6.

Sakaguchi, K., H. Sakamoto, et al. (1997). "Effect of phosphorylation on tetramerization of the tumor suppressor protein p53." J Protein Chem 16(5): 553-6.

Sakaguchi, T., A. Watanabe, et al. (1998). "Characteristics and clinical outcome of proximal-third gastric cancer." J Am Coll Surg 187(4): 352-7.

Sakamuro, D., P. Sabbatini, et al. (1997). "The polyproline region of p53 is required to activate apoptosis but not growth arrest." Oncogene 15(8): 887-98.

Sancar, A., L. A. Lindsey-Boltz, et al. (2004). "Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints." Annu Rev Biochem 73: 39-85.

Sandau, K., J. Pfeilschifter, et al. (1997). "Nitric oxide and superoxide induced p53 and Bax accumulation during mesangial cell apoptosis." Kidney Int 52(2): 378-86.

Sangiovanni, S. (1819). "Descrizione d'un particolare sisteina di organo cromoforo espansivo dermoideo e dei fenomeni ch'esse produce scopesto nei molluschi cefaloso." Giornale enciclopedico di Napoli 9: 1-15.

Schallreuter, K. (2005). "Oxidative stress in human epidermis." Giornale Italiano di Dermatologia e Venerologia 140 - N. 5(5): 505-14.

Schallreuter, K. U. (2005). Vitiligo. Autoimmune Diseases of the Skin. Pathogenesis, Diagnosis, Management. M. Hertl. Wien, Springer: 367-384.

Schallreuter, K. U., P. Bahadoran, et al. (2008). "Vitiligo pathogenesis: autoimmune disease, genetic defect, excessive reactive oxygen species, calcium imbalance, or what else?" Exp Dermatol 17(2): 139-40; discussion 141-60.

Schallreuter, K. U., S. Behrens-Williams, et al. (2003). "Increased epidermal functioning wild-type p53 expression in vitiligo." Exp Dermatol 12(3): 268-77.

Schallreuter, K. U., G. Büttner, et al. (1994). "Cytotoxicity of 6-biopterin to human melanocytes." Biochem Biophys Res Commun 204(1): 43-8.

Schallreuter, K. U., B. Chavan, et al. (2005). "Decreased phenylalanine uptake and turnover in patients with vitiligo." Mol Genet Metab 86 Suppl 1: S27-33.

Schallreuter, K. U., G. Chiuchiarelli, et al. (2006). "Estrogens can contribute to hydrogen peroxide generation and quinone-mediated DNA damage in peripheral blood lymphocytes from patients with vitiligo." J Invest Dermatol 126(5): 1036-42.

Schallreuter, K. U., S. M. Elwary, et al. (2004). "Activation/deactivation of acetylcholinesterase by H_2O_2: more evidence for oxidative stress in vitiligo." Biochem Biophys Res Commun 315(2): 502-8.

Schallreuter, K. U., N. C. Gibbons, et al. (2007). "Calcium-activated butyrylcholinesterase in human skin protects acetylcholinesterase against suicide inhibition by neurotoxic organophosphates." Biochem Biophys Res Commun 355(4): 1069-74.

Schallreuter, K. U., N. C. Gibbons, et al. (2007). "Hydrogen peroxide-mediated oxidative stress disrupts calcium binding on calmodulin: more evidence for oxidative stress in vitiligo." Biochem Biophys Res Commun 360(1): 70-5.

Schallreuter, K. U., N. C. J. Gibbons, et al. (2006). "Hydrogen peroxide mediated oxidation severely compromises the methionine sulfoxide A / thioredoxin reductase antioxidant defence system." J Invest Dermatol 126 Suppl 3: 77.

Schallreuter, K. U., S. Kothari, et al. (2008). "Regulation of melanogenesis--controversies and new concepts." Exp Dermatol 17(5): 395-404.

Schallreuter, K. U., J. Moore, et al. (1999). "In vivo and in vitro evidence for hydrogen peroxide (H_2O_2) accumulation in the epidermis of patients with vitiligo and its successful removal by a UVB-activated pseudocatalase." J Investig Dermatol Symp Proc 4(1): 91-6.

Schallreuter, K. U., J. Moore, et al. (2001). "Epidermal H_2O_2 accumulation alters tetrahydrobiopterin ($6BH_4$) recycling in vitiligo: identification of a general mechanism in regulation of all $6BH_4$-dependent processes?" J Invest Dermatol 116(1): 167-74.

Schallreuter, K. U., D. J. Tobin, et al. (2002). "Decreased photodamage and low incidence of non-melanoma skin cancer in 136 sun-exposed caucasian patients with vitiligo." Dermatology 204(3): 194-201.

Schallreuter, K. U., U. Wazir, et al. (2004). "Human phenylalanine hydroxylase is activated by H2O2: a novel mechanism for increasing the L-tyrosine supply for melanogenesis in melanocytes." Biochem Biophys Res Commun 322(1): 88-92.

Schallreuter, K. U. and J. M. Wood (2001). "Thioredoxin reductase - its role in epidermal redox status." J Photochem Photobiol B 64(2-3): 179-84.

Schallreuter, K. U., J. M. Wood, et al. (1991). "Low catalase levels in the epidermis of patients with vitiligo." J Invest Dermatol 97(6): 1081-5.

Schallreuter, K. U., J. M. Wood, et al. (1992). "Production of catecholamines in the human epidermis." Biochem Biophys Res Commun 189(1): 72-8.

Schallreuter, K. U., J. M. Wood, et al. (1996). "Increased monoamine oxidase A activity in the epidermis of patients with vitiligo." Arch Dermatol Res 288(1): 14-8.

Schallreuter, K. U., J. M. Wood, et al. (1994). "Regulation of melanin biosynthesis in the human epidermis by tetrahydrobiopterin." Science 263(5152): 1444-6.

Schallreuter, K. U., J. M. Wood, et al. (1994). "Defective tetrahydrobiopterin and catecholamine biosynthesis in the depigmentation disorder vitiligo." Biochim Biophys Acta 1226(2): 181-92.

Scherer, S. J., S. M. Maier, et al. (2000). "p53 and c-Jun functionally synergize in the regulation of the DNA repair gene hMSH2 in response to UV." Journal of Biological Chemistry 275(48): 37469-73.

Sengupta, S. and C. C. Harris (2005). "p53: traffic cop at the crossroads of DNA repair and recombination." Nat Rev Mol Cell Biol 6(1): 44-55.

Sevilla, L. M., R. Nachat, et al. (2007). "Mice deficient in involucrin, envoplakin, and periplakin have a defective epidermal barrier." J Cell Biol 179(7): 1599-612.

Shalbaf, M. (2009). More evidence for H_2O_2-mediated oxidative in vitiligo:increased DNA-damage. Department of Biomedical Science PhD thesis, University of Bradford

Shalbaf, M., N. C. Gibbons, et al. (2008). "Presence of epidermal allantoin further supports oxidative stress in vitiligo." Exp Dermatol 17(9): 761-70.

Shaulsky, G., N. Goldfinger, et al. (1991). "Nuclear localization is essential for the activity of p53 protein." Oncogene 6(11): 2055-65.

Shibanuma, M., T. Kuroki, et al. (1991). "Release of H_2O_2 and phosphorylation of 30 kilodalton proteins as early responses of cell cycle-dependent inhibition of DNA synthesis by transforming growth factor beta 1." Cell Growth Differ 2(11): 583-91.

Shieh, S. Y., J. Ahn, et al. (2000). "The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. [erratum appears in Genes Dev 2000 Mar 15;14(6):750]." Genes & Development 14(3): 289-300.

Shieh, S. Y., M. Ikeda, et al. (1997). "DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2." Cell 91(3): 325-34.

Shmueli, A. and M. Oren (2007). "Mdm2: p53's lifesaver?" Mol Cell 25(6): 794-6.

Sies, H. and E. Cadenas (1985). "Oxidative stress: damage to intact cells and organs." Philos Trans R Soc Lond B Biol Sci 311(1152): 617-31.

Siliciano, J. D., C. E. Canman, et al. (1997). "DNA damage induces phosphorylation of the amino terminus of p53." Genes Dev 11(24): 3471-81.

Slominski, A., D. J. Tobin, et al. (2004). "Melanin pigmentation in mammalian skin and its hormonal regulation." Physiol Rev 84(4): 1155-228.

Smith, M. L., I. T. Chen, et al. (1995). "Involvement of the p53 tumor suppressor in repair of u.v.-type DNA damage." Oncogene 10(6): 1053-9.

Song, X., A. Xu, et al. (2008). "Minocycline protects melanocytes against H_2O_2-induced cell death via JNK and p38 MAPK pathways." Int J Mol Med 22(1): 9-16.

Soussi, T., C. Caron de Fromentel, et al. (1990). "Structural aspects of the p53 protein in relation to gene evolution." Oncogene 5(7): 945-52.

Soussi, T. and P. May (1996). "Structural aspects of the p53 protein in relation to gene evolution: a second look." J Mol Biol 260(5): 623-37.

Spencer, J. D., B. Chavan, et al. (2005). "A novel mechanism in control of human pigmentation by β-melanocyte-stimulating hormone and 7-tetrahydrobiopterin." J Endocrinol 187(2): 293-302.

Spencer, J. D., N. C. J. Gibbons, et al. (2007). "Oxidative stress via hydrogen peroxide affects proopiomelanocortin-peptides directly in the epidermis of patients with vitiligo." J Invest Dermatol 127(2): 411-20.

Spencer, M. J., J. P. Vestey, et al. (1993). "Major histocompatibility class II antigen expression on the surface of epidermal cells from normal and ultraviolet B irradiated subjects." J Invest Dermatol 100(1): 16-22.

Srivastava, S., Z. Q. Zou, et al. (1990). "Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome [see comments]." Nature 348(6303): 747-9.

Stamler, J. S., S. Lamas, et al. (2001). "Nitrosylation. the prototypic redox-based signaling mechanism." Cell 106(6): 675-83.

Sterner, D. E. and S. L. Berger (2000). "Acetylation of histones and transcription-related factors. Washington, DC 64(2): 435-59.

Studier, F. W., A. H. Rosenberg, et al. (1990). "Use of T7 RNA polymerase to direct expression of cloned genes." Methods Enzymol 185: 60-89.

Subramanian, D. and J. D. Griffith (2002). "Interactions between p53, hMSH2-hMSH6 and HMG I(Y) on Holliday junctions and bulged bases." Nucleic Acids Res 30(11): 2427-34.

Sundaresan, M., Z. X. Yu, et al. (1995). "Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction." Science 270(5234): 296-9.

Szabo, C. (2003). "Multiple pathways of peroxynitrite cytotoxicity." Toxicol Lett 140-141: 105-12.

Tang, J. and G. Chu (2002). "Xeroderma pigmentosum complementation group E and UV-damaged DNA-binding protein." DNA Repair (Amst) 1(8): 601-16.

Tang, Y., W. Zhao, et al. (2008). "Acetylation is indispensable for p53 activation." Cell 133(4): 612-26.

Taylor, W. R. and G. R. Stark (2001). "Regulation of the G2/M transition by p53." Oncogene 20(15): 1803-15.

Tennant, R. W., J. E. French, et al. (1995). "Identifying chemical carcinogens and assessing potential risk in short- term bioassays using transgenic mouse models." Environ Health Perspect 103(10): 942-50.

Teoh, G., M. Urashima, et al. (1997). "MDM2 protein overexpression promotes proliferation and survival of multiple myeloma cells." Blood 90(5): 1982-92.

Thannickal, V. J., P. M. Hassoun, et al. (1993). "Enhanced rate of H2O2 release from bovine pulmonary artery endothelial cells induced by TGF-beta 1." Am J Physiol 265(6 Pt 1): L622-6.

Thomas, D. D., M. G. Espey, et al. (2002). "Protein nitration is mediated by heme and free metals through Fenton-type chemistry: an alternative to the NO/O2- reaction." Proc Natl Acad Sci U S A 99(20): 12691-6.

Thut, C. J., J. L. Chen, et al. (1995). "p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60." Science 267(5194): 100-4.

Tibbetts, R. S., K. M. Brumbaugh, et al. (1999). "A role for ATR in the DNA damage-induced phosphorylation of p53." Genes & Development 13(2): 152-157.

Tinel, A. and J. Tschopp (2004). "The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress." Science 304(5672): 843-6.

Tobin, D. J., N. N. Swanson, et al. (2000). "Melanocytes are not absent in lesional skin of long duration vitiligo." J Pathol 191(4): 407-16.

Tsatmali, M., P. Manning, et al. (1999). "alpha-MSH inhibits lipopolysaccharide induced nitric oxide production in B16 mouse melanoma cells." Ann N Y Acad Sci 885: 474-6.

Tsuchimoto, D., Y. Sakai, et al. (2001). "Human APE2 protein is mostly localized in the nuclei and to some extent in the mitochondria, while nuclear APE2 is partly associated with proliferating cell nuclear antigen." Nucleic Acids Res 29(11): 2349-60.

Umar, A., A. B. Buermeyer, et al. (1996). "Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis." Cell 87(1): 65-73.

Unger, T., T. Juven-Gershon, et al. (1999). "Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2." EMBO Journal 18(7): 1805-14.

Unger, T., T. Juven-Gershon, et al. (1999). "Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2." Embo J 18(7): 1805-14.

Vaganay-Juery, S., C. Muller, et al. (2000). "Decreased DNA-PK activity in human cancer cells exhibiting hypersensitivity to low-dose irradiation." Br J Cancer 83(4): 514-8.

van den Wijngaard, R. M., J. Aten, et al. (2000). "Expression and modulation of apoptosis regulatory molecules in human melanocytes: significance in vitiligo." Br J Dermatol 143(3): 573-81.

Vaziri, H., S. K. Dessain, et al. (2001). "hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase." Cell 107(2): 149-59.

Venot, C., M. Maratrat, et al. (1998). "The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression." Embo J 17(16): 4668-79.

Vertuani, S., A. Angusti, et al. (2004). "The antioxidants and pro-antioxidants network: an overview." Curr Pharm Des 10(14): 1677-94.

Vile, G. F. (1997). "Active oxygen species mediate the solar ultraviolet radiation-dependent increase in the tumour suppressor protein p53 in human skin fibroblasts." FEBS Lett 412(1): 70-4.

Villiotou, V. and G. Deliconstantinos (1995). "Nitric oxide, peroxynitrite and nitroso-compounds formation by ultraviolet A (UVA) irradiated human squamous cell carcinoma: potential role of nitric oxide in cancer prognosis." Anticancer Res 15(3): 931-42.

Vogelstein, B., D. Lane, et al. (2000). "Surfing the p53 network." Nature 408(6810): 307-10.

Vousden, K. H. and D. P. Lane (2007). "p53 in health and disease." Nat Rev Mol Cell Biol 8(4): 275-83.

Vousden, K. H. and X. Lu (2002). "Live or let die: the cell's response to p53." Nat Rev Cancer 2(8): 594-604.

Wadgaonkar, R. and T. Collins (1999). "Murine double minute (MDM2) blocks p53-coactivator interaction, a new mechanism for inhibition of p53-dependent gene expression [In Process Citation]." J Biol Chem 274(20): 13760-7.

Waga, S., G. J. Hannon, et al. (1994). "The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA [see comments]." Nature 369(6481): 574-8.

Waga, S. and B. Stillman (1998). "Cyclin-dependent kinase inhibitor p21 modulates the DNA primer-template recognition complex." Mol Cell Biol 18(7): 4177-87.

Walker, K. K. and A. J. Levine (1996). "Identification of a novel p53 functional domain that is necessary for efficient growth suppression." Proc Natl Acad Sci U S A 93(26): 15335-40.

Wang, R., A. Ghahary, et al. (1996). "Human dermal fibroblasts produce nitric oxide and express both constitutive and inducible nitric oxide synthase isoforms." J Invest Dermatol 106(3): 419-27.

Wang, X., D. Michael, et al. (2002). "p53 Activation by nitric oxide involves down-regulation of Mdm2." J Biol Chem 277(18): 15697-702.

Wang, X. W., M. K. Gibson, et al. (1995). "Abrogation of p53-induced apoptosis by the hepatitis B virus X gene." Cancer Res 55(24): 6012-6.

Wang, X. W., H. Yeh, et al. (1995). "p53 modulation of TFIIH-associated nucleotide excision repair activity." Nat Genet 10(2): 188-95.

Wang, Y., M. Reed, et al. (1993). "p53 domains: identification and characterization of two autonomous DNA- binding regions." Genes Dev 7(12B): 2575-86.

Warbrick, E. (2000). "The puzzle of PCNA's many partners." Bioessays 22(11): 997-1006.

Warren, J. B. (1994). "Nitric oxide and human skin blood flow responses to acetylcholine and ultraviolet light." Faseb J 8(2): 247-51.

Wasylyk, C., R. Salvi, et al. (1999). "p53 mediated death of cells overexpressing MDM2 by an inhibitor of MDM2 interaction with p53." Oncogene 18(11): 1921-34.

Waterman, M. J., E. S. Stavridi, et al. (1998). "ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins." Nat Genet 19(2): 175-8.

Westerhof, W. and M. d'Ischia (2007). "Vitiligo puzzle: the pieces fall in place." Pigment Cell Res 20(5): 345-59.

Westerhof, W. and K. U. Schallreuter (1997). "PUVA for vitiligo and skin cancer." Clin Exp Dermatol 22(1): 54.

Whibley, C., P. D. Pharoah, et al. (2009). "p53 polymorphisms: cancer implications." Nat Rev Cancer 9(2): 95-107.

White, E. (1996). "Life, death, and the pursuit of apoptosis." Genes Dev 10(1): 1-15.

Wilson, D. M., 3rd and L. H. Thompson (1997). "Life without DNA repair." Proc Natl Acad Sci U S A 94(24): 12754-7.

Wink, D. A., K. S. Kasprzak, et al. (1991). "DNA deaminating ability and genotoxicity of nitric oxide and its progenitors." Science 254(5034): 1001-3.

Wood, J. M., B. Chavan, et al. (2004). "Regulation of tyrosinase by tetrahydropteridines and H2O2." Biochem Biophys Res Commun 325(4): 1412-7.

Wood, J. M., H. Decker, et al. (2009). "Senile hair graying: H2O2-mediated oxidative stress affects human hair color by blunting methionine sulfoxide repair." Faseb J.

Wood, J. M., N. C. Gibbons, et al. (2008). "Computer simulation of heterogeneous single nucleotide polymorphisms in the catalase gene indicates structural changes in the enzyme active site, NADPH-binding and tetramerization domains: a genetic predisposition for an altered catalase in patients with vitiligo?" Exp Dermatol 17(4): 366-71.

Wood, J. M. and K. U. Schallreuter (2006). "UVA-irradiated pheomelanin alters the structure of catalase and decreases its activity in human skin." J Invest Dermatol 126(1): 13-4.

Xie, S., Q. Wang, et al. (2001). "Reactive oxygen species-induced phosphorylation of p53 on serine 20 is mediated in part by polo-like kinase-3." J Biol Chem 276(39): 36194-9.

Xu, B., S. T. Kim, et al. (2002). "Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation." Mol Cell Biol 22(4): 1049-59.

Xu, W., L. Liu, et al. (2000). "Nitric oxide upregulates expression of DNA-PKcs to protect cells from DNA-damaging anti-tumour agents." Nat Cell Biol 2(6): 339-45.

Yaar, M. and B. A. Gilchrest (1991). "Human melanocyte growth and differentiation: a decade of new data." J Invest Dermatol 97(4): 611-7.

Yang, A., M. Kaghad, et al. (1998). "p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities." Mol Cell 2(3): 305-16.

Yang, A., N. Walker, et al. (2000). "p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours." Nature 404(6773): 99-103.

Yang, G., G. Zhang, et al. (2006). "Expression Profiling of UVB Response in Melanocytes Identifies a Set of p53-Target Genes." J Invest Dermatol 126(11): 2490-506.

Yang, J., E. S. Bardes, et al. (1998). "Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1." Genes Dev 12(14): 2131-43.

Yew, P. R. and A. J. Berk (1992). "Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein." Nature 357(6373): 82-5.

Yonish-Rouach, E., D. Resnitzky, et al. (1991). "Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6." Nature 352(6333): 345-7.

Zhao, W., J. P. Kruse, et al. (2008). "Negative regulation of the deacetylase SIRT1 by DBC1." Nature 451(7178): 587-90.

Zhivotovsky, B. and G. Kroemer (2004). "Apoptosis and genomic instability." Nat Rev Mol Cell Biol 5(9): 752-62.

Zhou, J., J. Ahn, et al. (2001). "A role for p53 in base excision repair." Embo J 20(4): 914-23.